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THE MICRODETERMINATION OF SERINE AND ETHANOLAMINE IN PHOSPHOLIPIDE HYDROLYSATES*

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During an investigation (1) of the reactions of periodic acid with the water-soluble products of cephalin hydrolysis, a quantitative reaction of this reagent with ethanolamine was discovered. Titration by the arsenite method of Fleury and Paris (2) showed that 1 mole of periodate was reduced per mole of ethanolamine. It was found that, after the addition of dilute sodium hydroxide solution, 1 mole of ammonia could be recovered by distillation from the products of the reaction. The discovery, by Folch and Schneider (3), of a serine phospholipide in brain cephalin limited the use of this reaction for the determination of ethanolamine in phospholipides, since Nicolet and Shinn (4) had previously described the formation of ammonia by the reaction of periodate with serine. It was evident that, for cephalin hydrolysates containing serine and ethanolamine, the ammonia produced by the reaction of periodate measured both of these amino compounds. Other constituents of phospholipide hydrolysates, namely inositol and α -glycerophosphate, although they react with periodic acid (2, 5), do not interfere, since only compounds having a hydroxyl group and an amino group on adjacent carbon atoms produce ammonia.

A method with the microdiffusion technique described by Conway (6) was developed for measuring the ammonia formed by the action of periodate on serine and ethanolamine. The serine nitrogen was separately determined by the method of Van Slyke and Dillon (7) in which the carbon dioxide formed by the reaction of an α -amino acid (such as serine) with ninhydrin is measured. When the periodate nitrogen value of a phospholipide hydrolysate is corrected for the carboxyl nitrogen, the difference measures the ethanolamine nitrogen.

Method

Apparatus—The microdiffusion cells were constructed according to the directions of Bandemer and Schaible (8) from 50 mm. and 100 mm. Petri dishes. The small dishes were cemented in the center of the large dishes

* The data in this paper are taken from a thesis submitted to the Graduate School of the University of Rochester in partial fulfillment of the requirements for the degree of Doctor of Philosophy. A preliminary report of these data has appeared (1).

with paraffin. Square pieces of photographic glass, ground on one side with No. 300 carborundum powder and then greased with vaseline, served as covers for the larger dishes.

Reagents—

0.005 N hydrochloric acid containing a few drops of mixed indicator (0.08 gm. of methyl red and 0.02 gm. of methylene blue dissolved in 100 cc. of 1:1 alcohol-water solution and adjusted to a neutral gray color with 0.005 N sodium hydroxide).

0.005 N sodium hydroxide, carbonate-free.

0.05 M periodic acid. 11.4 gm. of H_5IO_6 from the G. Frederick Smith Chemical Company, Columbus, Ohio, in 1 liter of water.

Saturated potassium metaborate solution. 61.9 gm. of boric acid and 56.1 gm. of potassium hydroxide were ground together in a mortar, then carefully dissolved in 120 cc. of water.

*Procedure—*Introduce 1 to 5 cc. of 0.005 N acid by pipette or micro burette into the central chamber of the diffusion cell and 1 to 5 cc. of the water-soluble extract from an acid hydrolysate of phospholipide into the outer chamber. This sample should contain 0.05 to 0.3 mg. of total serine and ethanolamine nitrogen. Add 1 to 5 cc. of saturated potassium metaborate solution to the sample and mix by rocking the cell. Place the cover on the cell, sliding it so as to cover all but a small portion of the outer chamber. Pipette 1 cc. of 0.05 M periodic acid through this small opening and quickly slide the cover shut. Mix the contents of the outer chamber by rotation and allow diffusion to proceed at room temperature overnight. Titrate the standard acid remaining in the central chamber to a neutral gray color with 0.005 N base. Carry out a similar procedure, without the use of periodate, on a sample of the same size, to serve as a blank on the reagents and on any ammonium salts in the hydrolysate. The difference between the sample and the blank gives a measure of the serine + ethanolamine nitrogen. If desired, the serine nitrogen may be determined on another sample by the ninhydrin procedure (7) and the ethanolamine nitrogen obtained by difference.

*Calculation—*1 cc. of 0.005 N base = 0.070 mg. of periodate N.

EXPERIMENTAL

*Stability of Ammonia in Periodate Solutions—*1 cc. samples of a standard ammonium sulfate solution containing 0.022 mg. of nitrogen per cc. were treated with (a) 5 cc. of saturated potassium hydroxide; (b) 1 cc. of 10 N sulfuric acid and 5 cc. of saturated potassium hydroxide; (c) 1 cc. of 10 N sulfuric acid, 5 cc. of saturated potassium hydroxide, and 1 cc. of 0.05 M potassium periodate; and (d) 1 cc. of 10 N sulfuric acid, 1 cc. of 0.05 M potassium periodate, and after 15 minutes 5 cc. of saturated potassium hydroxide. Theoretical amounts of ammonia were recovered in all but the

last case, in which the recovery was low. These results indicated partial destruction of ammonia by warm acid periodate but none by warm alkaline periodate. It is possible that, on mixing acid and base, some parts of the acid solution become heated momentarily before being neutralized by the alkali.

Effect of Use of Various Alkalies to Free Periodate Ammonia—Table I shows the recovery of ammonia obtained when saturated solutions of po-

TABLE I

Microdiffusion of Ammonia from Reaction of Periodate with Ethanolamine and Serine

Ethan- olamine nitrogen	Serine nitrogen	Reagents added in order given to solution of sample in 1 cc. water; 20 hrs. diffusion at room temperature	Ammonia recovered, calculated as nitrogen	
			mg.	per cent
0.262		5 cc. saturated potassium hydroxide, 1 cc. 0.05 M periodic acid	0.00	0.0
	0.134	" "	0.015	11.2
0.262		1 cc. 0.05 M periodic acid; after 20 min., 5 cc. satu- rated potassium hydroxide	0.262	100.0
	0.134	" "	0.025	18.7
	0.134	1 cc. 1 N sulfuric acid, 1 cc. 0.05 M periodic acid; after 20 min., 5 cc. saturated potassium hydroxide	0.009	6.7
0.105		2 cc. saturated potassium carbonate, 1 cc. 0.05 M periodic acid	0.104	99.0
	0.118	" "	0.113	95.8
0.105		1 cc. 1 N sulfuric acid, 1 cc. 0.05 M periodic acid; after 20 min., 2 cc. saturated potassium car- bonate*		
0.102		5 cc. saturated potassium metaborate, 1 cc. 0.05 M periodic acid	0.102	100.0
	0.190	" "	0.189	99.4
0.218		1 cc. 5 N sulfuric acid, 1 cc. 0.05 M periodic acid; 10 cc. saturated potassium metaborate at once	0.217	99.5
	0.190	" "	0.190	100.0
0.218		1 cc. 5 N sulfuric acid, 1 cc. 0.05 M periodic acid; after 20 min. 10 cc. saturated potassium meta- borate	0.206	94.5

* Cover blew off owing to carbon dioxide.

tassium hydroxide, potassium carbonate, or potassium metaborate were used to free the ammonia produced by periodate in alkaline and acid solutions of ethanolamine and serine.¹ The reaction of periodate with ethanol-

¹ The ethanolamine was from a middle fraction collected on distillation of ethanolamine (Eastman Kodak, No. 1597). It gave a value of 99.65 per cent by electrometric titration to pH 4.8 with standard acid. The serine was prepared by recrystallization of *dl*-serine (Eastman Kodak, No. 4618) from 50 per cent alcohol followed by dehydration with absolute alcohol.

amine or serine was incomplete in potassium hydroxide solution. The use of potassium hydroxide to free ammonia from the reaction of ethanolamine or serine with periodate in acid solutions also gave very low results. Considerable heat was generated on addition of this alkali to acid solutions, but the recovery of ammonia was less than could be attributed to any mechanical loss due to the heat alone. The use of potassium carbonate with acid samples caused less generation of heat, but there was also a momentary liberation of carbon dioxide which caused the wall of the unit to be spattered with liquid. The reaction of serine with periodate in potassium carbonate solution required 58 hours for completion. Potassium metaborate, which is a reagent recommended by Conway (6) to avoid the heat generated in the outer chamber of a diffusion cell when strongly acid

TABLE II

Recovery of Serine and Ethanolamine Nitrogen Added to Phospholipide Hydrolysate

Experiment No.	Ethanolamine nitrogen	Serine nitrogen	Lecithin	Potassium dihydrogen phosphate	Inositol	Sodium glycerophosphate*	Acidity of solution	Ammonia recovered, calculated as nitrogen	
	mg.	mg.	mg.	mg.	mg.	mg.	N	mg.	per cent
1†	0.202	0.118	None	0.323	0.250	1.539	2	0.321	100.3
2	0.202	0.118	"	0.323	0.250	1.539	2	0.321	100.3
3	0.202	0.118	25.3	0.323	0.250	1.539	1.2	0.322	
4			25.3				1	0.004	
(3) - (4)								0.318	99.4

* Contained 52 per cent of the α -isomer.

† The samples of Experiments 1 and 2 contain all of the known water-soluble components of a cephalin hydrolysate.

solutions such as those from Kjeldahl digestions are treated with potassium hydroxide or potassium carbonate, gave excellent results with both ethanolamine and serine.

Effect of Periodate on Choline—Since all but the purest cephalin preparations contain lecithin, it was necessary to see whether the procedure with periodate in metaborate solution at room temperature had any effect on choline. Microdiffusion was used to measure the volatile bases freed from choline hydrochloride by (a) alkali alone, (b) periodate and alkali, and (c) alkali after treatment for 1 hour at room temperature with periodate in 1 N sulfuric acid solution. A small amount of base was freed by alkali alone. This amount was not increased by the presence of periodate or by pre-treatment with acid periodate.

Recovery of Serine and Ethanolamine Nitrogen Added to Phospholipide

Hydrolysate—The determination of serine and ethanolamine nitrogen by the use of periodate in metaborate solution was tested in the presence of all of the known water-soluble constituents of the hydrolysates of cephalin and lecithin. In these cases, the determinations were all carried out on acid samples because, in our experience, it has been found that hydrolysis of phospholipides must be carried on in acid solution in order to prevent loss of nitrogen from ethanolamine and serine. A known solution of ethanolamine and serine was made up containing all the known water-soluble constituents of a cephalin hydrolysate (potassium dihydrogen phosphate, inositol, sodium α -glycerophosphate, and sodium β -glycerophosphate) and analyzed for serine and ethanolamine nitrogen. This solution was then added to a lecithin hydrolysate² and the mixture analyzed. A cephalin hydrolysate could not be used, since it would contain an unknown amount of ethanolamine and serine. The results are given in Table II. As can be seen, the recovery of serine and ethanolamine nitrogen in the presence of all the known water-soluble constituents of a phospholipide hydrolysate was practically theoretical. 0.118 mg. of serine nitrogen was found by the ninhydrin method (7) on another aliquot of the combined solutions. Subtraction of this value from the 0.318 mg. of periodate nitrogen found by the diffusion procedure gave 0.200 mg. of ethanolamine nitrogen. These results are also in good agreement with theory.

DISCUSSION

The periodate nitrogen method described here gives a more specific measure of cephalin than does the nitrous acid method (10) used heretofore, since compounds other than serine and ethanolamine may react with nitrous acid. For example, Folch and Van Slyke (11) have reported that urea and amino acids are sometimes present in phospholipide preparations and that these compounds interfere with the measurement of cephalin nitrogen by nitrous acid. While it has been reported that hydroxylysine reacts with periodate to form ammonia (4), there has been no indication of the presence of this compound in phospholipide extracts.

Ramsay and Stewart (12) have reported the use of a microdiffusion method for the determination of ethanolamine with periodate and saturated potassium carbonate solution. They were able to obtain all of the theoretical ammonia from ethanolamine alone, but only 65 to 70 per cent of theory from ethanolamine added to phospholipide hydrolysates. It is believed that their low results may have been due to their use of potassium car-

² The lecithin was prepared from egg yolk by the procedure of Levene and Rolf (9). It was hydrolyzed by heating for 24 hours in 1 N hydrochloric acid, the fatty acids were filtered out, and aliquots of the filtrate were taken for the experiments shown in Table II.

bonate. When this alkali is used with an acid solution such as a phospholipide hydrolysate, there is some generation of heat and evolution of carbon dioxide, which may cause some mechanical loss and also some destruction of ammonia by hot acid periodate.

In addition to eliminating the difficulties mentioned above, the use of metaborate makes it possible to set free any preformed ammonia from the sample before addition of the periodate. This is impossible with potassium hydroxide or potassium carbonate because of the incomplete reaction of serine with periodate in the presence of these solutions.

SUMMARY

A method has been described for the determination of ethanolamine and serine nitrogen by microdiffusion of ammonia produced by periodate in a solution nearly saturated with potassium metaborate. It has been shown that by this method cephalin nitrogen can be measured in the presence of all the known components of phospholipide acid hydrolysates.

The author wishes to acknowledge his great indebtedness to Dr. W. R. Bloor for making this work possible.

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ON THE ACTIVATION OF CATALASE

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Kreke, Bartlett, and Smalt (1) have found that a product obtained by extracting bakers' yeast with alcohol (2), when present to the extent of 0.5 to 1.0 mg. of digest, accelerates the decomposition of hydrogen peroxide by crude catalase. They ran their determinations at 24° and expressed their results as per cent of hydrogen peroxide decomposed after different intervals of time. We have recalculated the results of one of their experiments as velocity constants, or K values, and present these in Table I. When extrapolated to zero time the K value obtained by using yeast extract

TABLE I
Velocity Constants for Catalase Activity

Digestion at 24°	K value with yeast extract	K value without yeast extract
min.		
5	0.062	0.030
10	0.072	0.030
15	0.064	0.028
20	0.052	0.029
25	0.043	0.026
30	0.038	0.025
35	0.035	0.024
40	0.030	0.025

is 0.074, while the value without yeast extract is less than one-half of this, or 0.032. We are unable to explain why yeast extract should be able to exert such a marked accelerating action upon catalase. Kreke *et al.* suggest that the yeast extracts contain iron complexes which may act to keep the catalase in the oxidized form. But since catalase is not reduced even by such a powerful reducing agent as sodium dithionite, we consider this hypothesis to be unlikely.

We have prepared three yeast extracts exactly as described by Kreke *et al.* and find that they do not accelerate the decomposition of hydrogen peroxide either by crystalline beef liver catalase or by crude rat liver catalase, either at 0° or at 20°. Here we have employed the method of von Euler and Josephson (3) and also a modification of the method of Jolles (4). Our results, expressed as K values extrapolated to zero time, are

shown in Table II. It is always possible, of course, that the yeast extracts prepared by us are somehow different from those prepared by Kreke *et al.*

Since catalase is rapidly inactivated by hydrogen peroxide, it is preferable to express catalase activity by extrapolating to zero time, and it is best to run the determinations at 0°. *K* values determined after catalase has reacted with hydrogen peroxide for some minutes can be used to show whether or not some added substance is capable of protecting the catalase

TABLE II
Effect of Yeast Extract on Different Catalases

Catalase used	Method of determination	Temperature	<i>K</i> value with yeast fraction	<i>K</i> value without yeast fraction
		°C.		
Crystalline beef liver	von Euler and Josephson	0	0.046	0.046
" " "	" " " "	0	0.053	0.055
" " "	" " " "	20	0.057	0.056
" " "	" " " "	0	0.061	0.067
" " "	" " " "	0	0.039	0.045
Crude rat liver	" " " "	0	0.014	0.016
" " "	" " " "	20	0.040	0.043
" " "	Jolles	0	0.044	0.047
" " "	"	0	0.029	0.026

TABLE III
Effect of Yeast Extract on Activation and Destruction of Catalase
Temperature 24°; ground rat liver as source of catalase.

Digestion time	<i>K</i> value without yeast extract	<i>K</i> value with yeast extract
min.		
5	0.036, 0.039, 0.038	0.035, 0.038, 0.040
10	0.034, 0.035, 0.034	0.038, 0.038, 0.039
15	0.029, 0.033, 0.031	0.035, 0.037, 0.038
20	0.029, 0.031, 0.029	0.036, 0.035, 0.036

from destruction by peroxide. Our data, given in Table III, are from triplicate determinations carried out exactly like those of Kreke *et al.*, both with and without the addition of yeast extract. The data show plainly that while the yeast extract does not activate the catalase it does protect it from destruction, since values after 20 minutes are higher when the extract has been added than in its absence.

SUMMARY

Contrary to the claim of Kreke, Bartlett, and Smalt, our yeast extracts did not increase the activity either of crude or of crystalline catalase. The

extracts did protect crude rat liver catalase from inactivation by hydrogen peroxide at 24°.

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THE CYTOCHROME *c*-CYANIDE COMPLEX

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In the course of studies involving the reduction of ferricytochrome *c* by the succinic dehydrogenase system, it was observed that in the presence of cyanide there is a progressive decrease in the amount of reducible cytochrome *c*. This action of cyanide on cytochrome *c* had previously been reported by Potter (1), together with other evidence indicating the formation of a ferricytochrome *c*-cyanide complex, particularly a shift in the absorption maximum from 5300 to 5350 Å.

As a result of their spectroscopic observations, Keilin and Hartree (2, 3) had concluded that cytochrome *c*, unlike other ferriheme proteins, did not form a cyanide complex, and until the work of Potter this conclusion was generally accepted. Their failure to observe the shift in the position of the absorption maximum may be attributed to the fact that the shift is too small to be detected by direct visual examination. Keilin and Hartree also noted that the reduced bands of cytochrome *c* appeared when cyanide was added to a crude yeast or tissue preparation. This observation, however, is not inconsistent with the existence of a ferricytochrome *c*-cyanide complex, since the rapidity of reduction of cytochrome *c* in such preparations will preclude any appreciable reaction between cyanide and ferricytochrome *c*.

In the present investigation, the change in spectrum of oxidized cytochrome *c* in the presence of cyanide has been confirmed. The kinetics of the reaction between cyanide and ferricytochrome *c* have been studied in detail, and constants for the reaction derived therefrom. The results of these kinetic studies offer an explanation for the sluggish nature of the reaction, the relatively high concentrations of cyanide required, and the apparent irreversibility noted by Potter. In addition, a new band is described for ferricytochrome *c* at 6925 Å, which may be observed in concentrated solution and which disappears on addition of cyanide or reduction.

EXPERIMENTAL

Cytochrome c—This was prepared from calf heart by the method of Keilin and Hartree (2), except that the cytochrome solution was finally dialyzed against distilled water and evaporated to a dry powder from the frozen state. The preparation contained 0.24 per cent Fe. By repeating

the final $(\text{NH}_4)_2\text{SO}_4$ -trichloroacetic acid precipitation, the Fe content was raised to 0.35 per cent.

Recently Keilin and Hartree (4) have described a method whereby the Fe content of their cytochrome *c* preparation was increased to 0.43 per cent by precipitation with $(\text{NH}_4)_2\text{SO}_4$ at pH 10.0. By this procedure a preparation was obtained which contained 0.41 per cent Fe.¹

The kinetic measurements described below were obtained with the cytochrome *c* preparation containing 0.24 per cent Fe. Since the extinction coefficients based on Fe content are not affected by the further purification (4, 5), these results are considered to apply as well to pure cytochrome *c*. The absorption spectra of concentrated solutions in the red region are compared for the various preparations described.

To insure complete oxidation of the cytochrome solutions, these were brought to pH 4.0 with 0.1 N HCl and aerated before neutralization and further dilution for each experiment.

Succinic Dehydrogenase—This was prepared from rat heart by the method of Stotz and Hastings (6), except that the washings with water were omitted. From 3.5 gm. of rat heart about 4 cc. of a succinic dehydrogenase suspension were obtained. These preparations were stored in the refrigerator and used for 2 weeks.

Cyanide Solutions—0.15 M KCN solutions were freshly prepared for each experiment by dissolving 500 mg. in water and neutralizing with 1.0 N HCl. 5.0 cc. of 0.5 M phosphate buffer, pH 7.4, were then added and the total volume brought to 50 cc. Appropriate dilutions in 0.05 M phosphate buffer were prepared as required.

Succinate Solutions—2.5 gm. of sodium succinate + $6\text{H}_2\text{O}$ were dissolved in 5.0 cc. of 0.5 M phosphate buffer, pH 7.4, and diluted to 50 cc. to give a 0.185 M solution.

Fe Determinations and Dry Weights—Fe contents of the cytochrome preparations were determined by the method of Delory (7). Dry weights of the preparations containing salt were determined by the ignition method described by Keilin and Hartree (4).

Spectrophotometry and Colorimetry—The cytochrome solutions were analyzed with the Beckman spectrophotometer at 5500 Å after reduction with $\text{Na}_2\text{S}_2\text{O}_4$. The extinction coefficient based on Fe content at this wavelength was found to agree with that given by Theorell (8), although for ferricytochrome *c* and for ferrocytochrome *c* at 5200 Å the extinction coefficients were somewhat lower. The absorption spectra were also deter-

¹ With the addition of $\frac{1}{2}$ volume of NH_3 , as prescribed by Keilin and Hartree, the pH was 9.6, rather than 10.0. However, with larger volumes of NH_3 , the $(\text{NH}_4)_2\text{SO}_4$ saturation was reduced and no purification was obtained. Failure to obtain a purer preparation may be due to the low pH value.

mined with the Beckman spectrophotometer, with a slit width of 40 Å in the blue and green and 50 to 60 Å in the red region.

The kinetic measurements were made with the aid of a photoelectric colorimeter and a 5490 Å filter transmitting between the extremes of 5350 and 5700 Å. With this filter the proportionality of density to concentration was obtained for ferricytochrome *c* over the entire range, but for reduced cytochrome *c* only when the concentration was maintained below 2.0×10^{-5} M, with a 1.0 cm. cell. Within this range the conversion of ferricytochrome *c* to ferrocytochrome *c* followed the equation

$$(1) \quad C(\text{ferricytochrome } c) = \frac{\left(\log \frac{I_0}{I}\right)_{\text{Fe}^{++}} - \left(\log \frac{I_0}{I}\right)_{\text{Fe}^{+++}}}{0.069} \times 10^{-4}$$

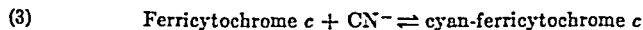
where *C* (ferricytochrome *c*) is the concentration in moles per liter, *I*₀ the intensity of incident light, and *I* the intensity of transmitted light. Fe⁺⁺⁺ and Fe⁺⁺ represent the solution before and after the ferricytochrome *c* was converted to ferrocytochrome *c* by enzymatic reduction.

Although cyan-ferricytochrome *c* is not appreciably reduced by succinic dehydrogenase over relatively long periods of time, as observed by Potter, it is readily reduced by Na₂S₂O₄. For the conversion of cyan-ferricytochrome *c* to ferrocytochrome *c* the following equation was found to apply.

$$(2) \quad C(\text{cyan-ferricytochrome}) = \frac{\left(\log \frac{I_0}{I}\right)_{\text{Fe}^{++}} - \left(\log \frac{I_0}{I}\right)_{\text{Fe}^{+++}}}{0.061} \times 10^{-4}$$

Both Equations 1 and 2 are valid for solutions in which no other changes in light absorption are taking place.

In the determination of the rate and equilibrium constants for the reaction



the concentrations of free ferricytochrome *c* and of the cyanide complex were determined from the change in optical density produced by successive additions of excess succinate-succinic dehydrogenase and Na₂S₂O₄, Equations 1 and 2 respectively being applied. Since the total cytochrome concentration was given by the final value, these results served to check each other. Suitable corrections were made in each case for turbidity contributed by the succinic dehydrogenase added.

Rate of Reaction

Ferricytochrome *c* was incubated with various concentrations of cyanide at several temperatures. At intervals during the incubation period ali-

quots were removed from the mixture and treated with a succinate-succinic dehydrogenase mixture. The quantity of enzyme added was sufficient to reduce completely the ferricytochrome *c* present in less than 0.2 minute. The results of a typical experiment are given in Fig. 1. 1.52×10^{-5} M ferricytochrome *c* was incubated with 0.0045 M, 0.015 M, and 0.03 M KCN in a total volume of 10.0 cc. in 0.05 M phosphate buffer, pH 7.4. At the time intervals indicated by the points on the curves, 1.0 cc. quantities of the cytochrome-cyanide mixtures were added to 0.5 cc. of a solution of

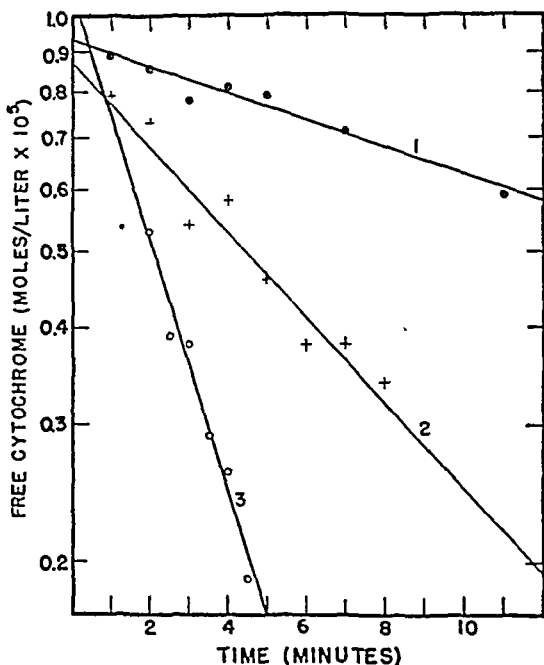


Fig. 1. The rate of formation of cyan-ferricytochrome *c* at 24°, pH 7.4. Curve 1, KCN 0.0045 M, CN⁻ 7.9×10^{-5} M; Curve 2, KCN 0.015 M, CN⁻ 26×10^{-5} M; Curve 3, KCN 0.03 M, CN⁻ 53×10^{-5} M.

0.037 M succinate in 0.05 M phosphate buffer, pH 7.4, containing 0.005 cc. of succinic dehydrogenase. Optical densities were measured after reduction by enzyme and again after addition of about 2 mg. of dry $\text{Na}_2\text{S}_2\text{O}_4$. The pH of each solution was determined at the end of the reaction. At the beginning and end of the experiment 1.0 cc. samples were diluted with 0.5 cc. of water and the density determined in order to establish the base value for computation by Equation 1. The values for intermediate times were obtained by interpolation.

As shown in Fig. 1, the rate of combination of cyanide with ferricyto-

chrome *c* follows the equation for a reaction of the first order

$$\frac{d \log_{10} (\text{ferricytochrome } c)}{dt} = k(\text{CN}^-)$$

from which it may be concluded that the reaction involves 1 mole of ferricytochrome *c* and 1 mole of CN^- , as represented in Equation 3. The specific reaction velocity constant, *k*, is obtained from the slope of the curves in Fig. 1.

$$k = \frac{2.303}{(\text{CN}^-)} \times \frac{d \log_{10} (\text{ferricytochrome } c)}{dt} \text{ liter mole}^{-1} \text{ min.}^{-1}$$

In Table I are listed the values obtained for *k* at various temperatures.

TABLE I

Specific Reaction Velocity Constants for Formation of Cyan-ferricytochrome c
k = liter mole⁻¹ min.⁻¹

2°	24°	37°
16.8	590	3440
15.8	540	4160
20.1	480	3840
17.0	550	
15.0	500	
17.4	580	
	540	
	590	
Average.....17.0	550	3810

From the effect of temperature on the rate of the reaction, the heat of activation, *A*, may be calculated by means of the Arrhenius equation

$$\frac{2.303d \log_{10} k}{dT} = \frac{A}{RT^2}$$

When the values of $\log_{10} k$ are plotted against $1/T$, the reciprocal of the absolute temperature, the heat of activation computed from the slope of the line obtained is 26,100 calories per mole.

Equilibrium Constant

The procedure for the determination of the equilibrium constant

$$K = \frac{(\text{ferricytochrome } c)(\text{CN}^-)}{(\text{cyan-ferricytochrome } c)}$$

was similar to that for the determination of the reaction velocity except that lower concentrations of cyanide and longer incubation times were used. To reduce loss of cyanide during the reaction, the cyanide-cytochrome mixtures were transferred to 10 cc. syringes fitted with hypodermic needles immediately after preparation and the needle sealed by insertion into a cork stopper. Samples were delivered through the needle as required. Since there was no necessity for a very rapid enzymatic reduction, only one-fifth

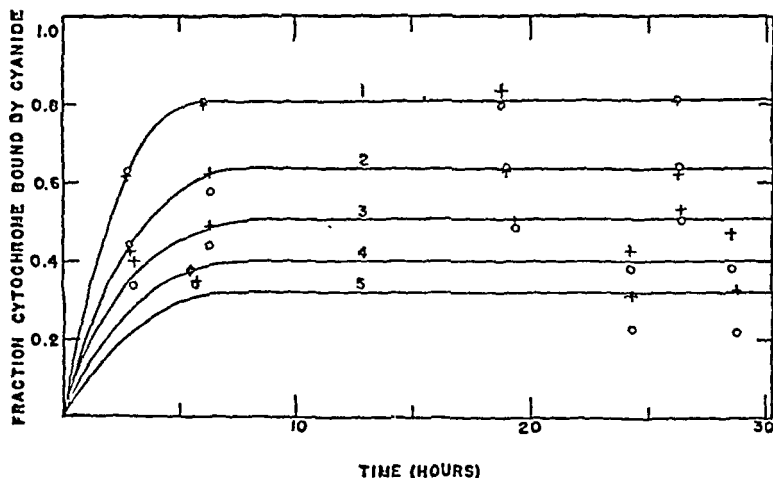


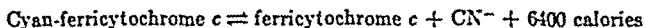
FIG. 2. Equilibrium between ferricytochrome *c* and cyanide at 27°. Curve 1, KCN 7.5×10^{-4} M, pH 7.32, CN⁻ 11.2×10^{-6} M; Curve 2, KCN 3.0×10^{-4} M, pH 7.34, CN⁻ 4.7×10^{-6} M; Curve 3, KCN 1.5×10^{-4} M, pH 7.32, CN⁻ 2.24×10^{-6} M; Curve 4, KCN 1.0×10^{-4} M, pH 7.38, CN⁻ 1.69×10^{-6} M; Curve 5, KCN 0.5×10^{-4} M, pH 7.40, CN⁻ 0.88×10^{-6} M. Circles represent values calculated from enzyme-reducible cytochrome; crosses represent values obtained by reduction of cyan-ferricytochrome *c* by Na₂S₂O₄.

as much enzyme was used in order to reduce the size of the turbidity correction. At the time of each determination, control samples were diluted with water and the densities measured.

The results of a typical experiment are given in Fig. 2. The time required to reach equilibrium varied with the temperature but in most cases at least two points were obtained after equilibrium was reached. In the experiments at 2° equilibrium had not been reached after 3 days, and the curves were extrapolated to determine the asymptotic equilibrium values.

Determinations of the equilibrium constant at the various temperatures are summarized in Table II. From the slope of the curve obtained by plotting $\log_{10} K$ against the reciprocal of the absolute temperature, the heat of the reaction is calculated to be -6400 calories per mole, in the range of

temperature covered. The equation for the dissociation of cyan-ferricytochrome *c* may therefore be written



In order to establish that the reaction involves cyanide ion, rather than undissociated HCN, the measurement of the equilibrium constant was repeated at pH 8.0, at which the HCN is approximately 4 times as dissociated as at pH 7.4. The results, computed on the basis of cyanide ion, were in agreement with those obtained at pH 7.4, whereas constants computed on the basis of the KCN concentration were only one-fifth as large. Similar results were obtained from the effect of pH on the reaction velocity. From these experiments it may be concluded that the reaction is correctly represented by Equation 3.

TABLE II

Dissociation Constant of Cyan-ferricytochrome c

K = moles per liter, average values.

Temperature	<i>K</i> × 10 ⁴
°C.	
2.5	6.3
15.0	4.2
27.0	2.5
37.0	2.0

While no quantitative measurements were made with cyan-ferricytochrome *c* as the starting material, the reversibility of the reaction was established by dialysis after complete conversion to the complex. The recovered ferricytochrome was readily reducible by the succinic dehydrogenase system.

Absorption Spectra

As was previously indicated, the present study has confirmed the shift in absorption maximum reported by Potter. The change produced by cyanide in this region is, however, small compared to the decrease in absorption observed in the red region. Here ferricytochrome *c* has a well defined band at 6925 Å, which is absent in the cyan derivative and in ferrocytochrome *c* (Fig. 3) and also shows a hump at 6400 Å.

From the spectra obtained with the various cytochrome *c* preparations it is evident that the band at 6925 Å increases in intensity as the cytochrome is purified. The hump at 6400 Å, on the other hand, tends to diminish with purification. It is present as well in the spectra of cyan-

ferricytochrome c and ferrocyanochrome c. It is, therefore, considered likely that this hump represents absorption due to some impurity, while the 6925 Å band is attributable to ferricytochrome c itself. This conclusion is

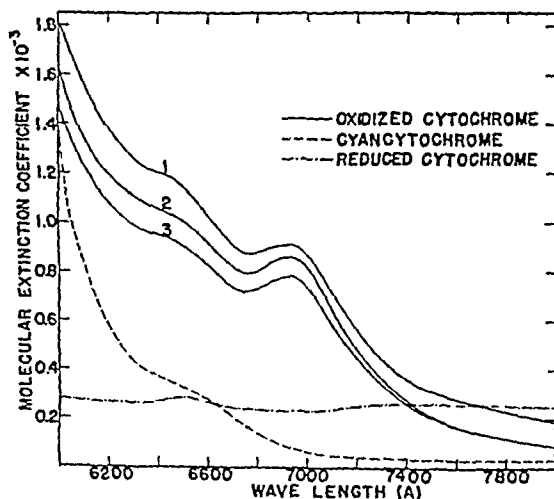


FIG. 3. The absorption spectra of cytochrome c derivatives in concentrated solution. The molecular extinction coefficients are based on Fe content. Curve 1, Fe 0.24 per cent; Curve 2, Fe 0.35 per cent; Curve 3, Fe 0.41 per cent. The curves for reduced and cyan-cytochrome c were determined with 0.41 per cent Fe cytochrome.

TABLE III
Properties of Cyanide-Ferricytochrome c Reaction

	25°	37°
Specific reaction velocity constant for formation, liter min. ⁻¹ mole ⁻¹	660	3810
Half time of formation of complex in 10 ⁻³ M KCN, pH 7.4, min.	60	10
Dissociation constant, moles per l. × 10 ⁶	2.6	1.9
Fraction of cytochrome as complex at equilibrium in 10 ⁻³ M KCN, pH 7.4	0.87	0.93
Specific reaction velocity constant for dissociation, min. ⁻¹ × 10 ³	1.7	7.2
Half time of dissociation in absence of cyanide, min.	407	96
Heat of dissociation, calories		-6,400
“ “ activation “		26,100

borne out by the kinetic behavior of the absorption at these wave-lengths in the presence of cyanide. 2.9 cc. of a 2.06×10^{-4} M solution of ferricytochrome c in 0.05 M phosphate buffer, pH 7.4, were treated with 0.1 cc. of 0.15 M KCN and the change in absorption with time followed at 6900 and 6400 Å. At 6900 Å there was a progressive decrease in density which, when

converted to ferricytochrome *c* concentration and plotted according to Equation 4, gave a linear relationship and a rate constant in excellent agreement with those obtained by enzymatic reduction. At 6400 Å, on the other hand, the density fell initially to a new value, after which the behavior coincided with that observed at 6900 Å. The magnitude of this initial decrease was such as to eliminate the hump completely and give a smooth curve between 6200 and 6700 Å. From this observation it might be concluded that the hump at 6400 Å is due to an impurity which combines with cyanide more rapidly than does ferricytochrome *c*. This conclusion, however, is not in accord with the presence of the hump in the spectrum of cyan-ferricytochrome *c*, and the nature of the 6400 Å hump must therefore remain in doubt.

One other observation of interest is presented in connection with the absorption measurements. When ferricytochrome *c* containing 0.24 or 0.35 per cent Fe is treated with cyanide, formation of the cyanide derivative is accompanied by the appearance of a precipitate which must be removed by centrifugation before the cyan-ferricytochrome spectrum is determined. No precipitate is formed, however, when ferricytochrome *c* containing 0.41 per cent Fe combines with cyanide. Apparently cyanide is capable of splitting off a fraction from that form of cytochrome *c* containing 0.34 per cent Fe in a manner similar to the dissociation at alkaline pH (3).

In Table III, the important properties of the cyanide-ferricytochrome *c* reaction are summarized.

DISCUSSION

In addition to Potter's work, there is a considerable amount of indirect evidence for the action of cyanide at some locus other than cytochrome oxidase. Stannard (9) found a difference in the kinetics of the cyanide sensitivity of the respiration of resting and active muscle and showed that the resting respiration was not inhibited by azide. To account for these findings he postulated the existence of an alternative cyanide-sensitive respiration mechanism. Winzler (10), on the basis of the kinetic behavior of yeast respiration in the presence of cyanide, has also concluded that a second cyanide-sensitive carrier is involved in addition to cytochrome oxidase. The possibility is suggested that this carrier is cytochrome *c*. However, in view of the fact that the effect of cyanide occurs "within a few seconds" this possibility appears unlikely. Winzler has calculated the dissociation constant of this cyanide complex and found it to be 3.7×10^{-5} M NaCN. Assuming the pH within the yeast cell to be the same as that of the medium, this would give a value of 3×10^{-3} for the dissociation constant computed on the basis of cyanide ion, indicating a much more stable complex than cyan-ferricytochrome *c*.

From the results of the present investigation, it may be concluded that

the cyanide-cytochrome complex will, because of the inhibition by cyanide of cytochrome oxidase, contribute little to the effect of cyanide on respiration. As a result of the immediate and complete inhibition of the oxidase by cyanide, cytochrome *c* will rapidly be reduced by the dehydrogenase systems, before appreciable combination with cyanide occurs. Only if there should exist an oxidation mechanism for cytochrome *c* less cyanide-sensitive than cytochrome oxidase, will the possibility arise for the *in vivo* formation of cyan-ferricytochrome *c*. Perhaps the existence of such an oxidation mechanism will account for the observations of Stannard and Winzler.

The authors are indebted to Dr. Dean Burk and Dr. F. S. Brackett for helpful suggestions, and to Mr. Loyall G. Goff for technical assistance.

SUMMARY

1. Ferricytochrome *c* and cyanide ion combine in equimolar proportions to form a cyan-ferricytochrome *c* complex.
2. The kinetics of the reaction have been studied. The reaction is sluggish but results in the formation of a moderately stable complex. The heat of activation for formation of the complex is 26,100 calories. The heat of dissociation is -6400 calories.
3. The shift in absorption maximum of ferricytochrome *c* in the presence of cyanide as reported by Potter has been confirmed. A new band is described for ferricytochrome *c* at 6925 Å, which disappears on addition of cyanide or reduction.
4. The physiological significance of the formation of the cyan-ferricytochrome *c* complex is discussed.

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BIOPHYSICAL STUDIES OF BLOOD PLASMA PROTEINS

IV. SEPARATION AND PURIFICATION OF A NEW GLOBULIN FROM NORMAL HUMAN PLASMA*

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The antibodies of various animal species immunized to different antigens have been shown to possess electrophoretic mobilities of either γ -globulin or of a component moving with the approximate velocity of fibrinogen (1-5). In the course of studies on the separation and removal of γ -globulin (6) from human blood plasma protein pastes prepared by ethanol precipitation and known as Fractions II + III and III-1 (7), it was noticed that a protein possessing an electrophoretic mobility comparable to that of fibrinogen was always present in the residues resulting after such extractions. The work to be reported here describes the concentration and purification of this additional, non-fibrinogen, normal human blood plasma protein. For reasons to be disclosed we have designated it as γ_1 -globulin. On this basis then, the normal serum γ -globulin is to be referred to as γ -globulin. Moreover, it is a consequence of these studies that the yield of the normal serum γ -globulin from the original Fraction II + III pastes is now increased to approximately 95 per cent.

EXPERIMENTAL

The general techniques for the fractionation of normal serum γ -globulin were used. The starting material was that portion of Fraction II + III (8) which precipitated at pH 5.1, ionic strength 0.01, ethanol concentration 15 to 17 per cent, and temperature -6° , as carried out in the separation of normal human serum globulins (6). This residue was suspended in distilled water at 0° and a study was made of the variables salt, pH, and ethanol concentration in order to find the conditions necessary to effect solution of the γ_1 -globulin with precipitation of the remaining proteins. In all fractionations, the temperatures were maintained within 1° of the freezing point of the system; the pH measurements were carried out at 25° by means of a glass electrode.

It was found that a set of conditions which was necessary to maintain the major portions of the γ_1 -globulin in solution with simultaneous precipitation

* This work has been carried out under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Wisconsin.

of the lipide-containing β -globulins resulted in solution of the γ_2 -globulin as well. Thus, it became necessary to find conditions to resolve an initial fraction consisting of these two γ -globulins into the two constituent proteins. The progress of the fractionation was evaluated (1) by means of dry weight analysis of the protein fractions and (2) by a study of their electrophoretic patterns. The electrophoretic analyses were carried out at pH 8.6, in a barbiturate-citrate buffer of ionic strength 0.088, in which the sodium barbiturate ions contributed 48 per cent of the ionic strength. The moving boundaries were recorded with the usual Tiselius assembly, by the diagonal knife-edge schlieren method. The duration of an experiment was 2 hours with a potential gradient of approximately 10 volts per cm.

Results

The small amount of γ_1 -globulin and its relation to the other plasma proteins are shown by Fig. 1, which is an electrophoresis record of human

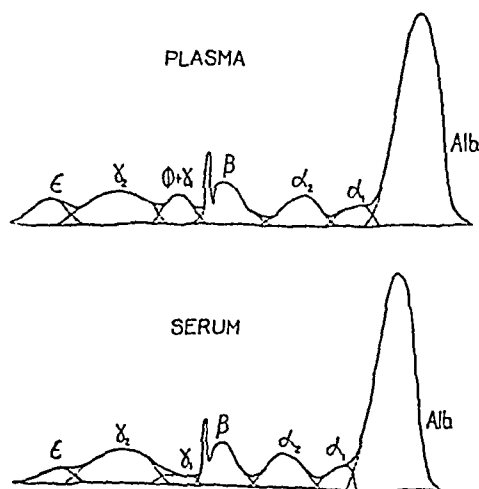


FIG. 1. Electrophoretic diagram of human blood plasma and serum from the same individual.

plasma and serum from the same individual. In plasma diagrams this component is commonly estimated as fibrinogen but it can be clearly seen from the serum pattern that another protein is present. Cohn *et al.* (8) indicate that such a protein represents 3 per cent of the total plasma proteins.

The Fraction II + III contains the major portion of the serum γ_2 - and γ_1 -globulins. After the removal of a large portion (75 to 80 per cent) of the γ_2 -globulin by the use of the procedure described in Paper III of this

series (6), there is formed a new protein residue which has the electrophoretic composition shown in Fig. 2. Although the residual fibrinogen in Fraction II + III has been denatured by prior chemical treatments, it is possible that a portion of the area indicated as γ_1 -globulin may consist of a lysis product of fibrinogen, such as the α -fibrinogen derivative of Seegers *et al.* (9). However, as judged by the criterion of fibrin formation, no fibrinogen is present. Such a consideration makes difficult an evaluation of over-all yield of the γ_1 -globulin in a recovery process.

The main difficulty that had to be overcome in the recovery of γ_1 -globulin was the removal, by precipitation, of the lipide-containing β -globulins. Complete separation of the β -globulin is difficult, and is impractical, if suitable yields of γ_1 -globulin are desired.

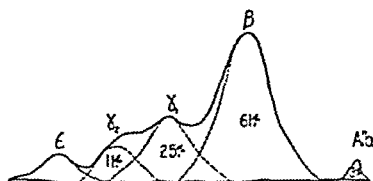


FIG. 2. Electrophoretic diagram of protein paste used as the starting material in fractionations.

Thus, the primary object of our research was the removal by precipitation of such β -globulins from the protein solutions; these steps will be referred to as Precipitations I in the report. As γ -globulins must be also removed from the supernatants by precipitation procedures, these operations will be described as Precipitations II.

Initial experiments indicated that, at minimal ionic strengths (about 0.002), the major portions of the β -globulins could be removed in aqueous solution at pH 5.1 with little loss of γ_1 -globulin. The use of lower pH values resulted in failure to precipitate a large part of the β -globulins, but the use of higher pH values, while removing as much of this globulin, resulted in high losses of γ_1 -globulin by precipitation. Hence the initial step in all experiments was to remove the globulins (largely β -globulins) insoluble at pH 5.1, and ionic strength 0.002, even without the addition of alcohol (Precipitation Ia). The presence of small amounts of ethanol (approximately 0.5 per cent) at this point was due to the alcohol content of the starting protein pastes (about 13 per cent by volume). The γ -globulins present in these supernatant solutions, when precipitated at pH 7.0, ionic strength 0.01, and ethanol concentration 20 per cent (Precipitation IIa), showed an electrophoretic composition as indicated in Fig. 3.

It might be well to state at this point that the electrophoretic analysis of protein mixtures such as γ_1 - and γ_2 -globulins is somewhat arbitrary, because the resolution is poor. In a future report we will deal with this problem; namely, the ability to assay mixtures of known γ_1 - and γ_2 -globulin content by electrophoresis.

Attempts to remove more β -globulins (Precipitation Ib) by adjustments of the pH of the supernatant solutions resulting from Precipitation Ia were not satisfactory. Although products containing less than 10 per cent β -globulins were regularly obtained, such attempts resulted in poor yields of the desired γ_1 -globulin. The results of some experiments of this kind are

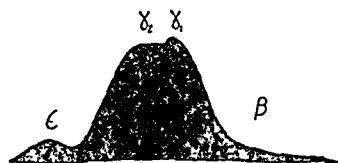


FIG. 3. Electrophoretic diagram of protein in the starting material soluble at pH 5.1, $\mu = 0.002$.

TABLE I
Effect of pH Adjustments on Precipitation Step Ib

Experiment	Pptn. Ib		Pptn. IIa, 20 per cent ethanol		Yield	Electrophoretic analysis		
	pH	μ	pH	μ		γ_2 -Globulin	γ_1 -Globulin	β -Globulin
						per cent	per cent	per cent
A	5.51	0.0014	7.10	0.0067	10.2	30	64	6
B	6.05	0.0017	7.12	0.0061	7.6	39	54	7
C	6.55	0.0030	7.20	0.0069	6.0	30	64	6
D	7.20	0.0150	7.10	0.011	15.4	26	65	9

collected in Table I. Except when otherwise indicated, the data in Tables I to IX refer to fractionations carried out on 200 gm. portions of protein pastes of the electrophoretic composition shown in Fig. 2. This corresponds to 50 to 60 gm. of dry protein.

Since adjustments of pH of the supernatants following the Precipitation Ia step were not successful in removing substantial amounts of the β -globulins still in solution, other means to accomplish the purification were attempted. The use of added ethanol to attain such a separation was studied.

While such experiments at various ethanol concentrations tended to show differences in yield rather than an improved separation of the β -globulins, it was indicated that an ethanol concentration of 5 to 10 per cent

at this point (Precipitation Ib) could be used satisfactorily. These results are shown in Table II.

Occasionally, albumin was a contaminant of the γ_1 - and γ_2 -globulin product, along with the β -globulins. To keep the albumin and possibly a portion of the β -globulin in solution while precipitating the γ -globulins, the effects of the use of higher pH values were investigated. As shown by the results in Table III, the use of pH 7.7, with an ethanol concentration of 20 per cent, was instrumental in giving an improved product (Precipitation IIa).

TABLE II

Effect of Ethanol Concentration on Precipitation Step Ib at pH 5.09 and μ 0.0012

Experiment	Ethanol concentration for Pptn. Ib	Ionic strength for Pptn. IIa, pH 7.0, 30 per cent ethanol	Yield	Electrophoretic analysis			
				γ_2 -Globulin	γ_1 -Globulin	β -Globulin	Albumin
	per cent		gm.	per cent	per cent	per cent	per cent
A	4.8	0.005	17.0	25	66	9	0
B	7.4	0.014	16.0	16	76	8	0
C	9.9	0.005	13.2	23	62	8	7

TABLE III

Effect of pH on Precipitation Step IIa

For Precipitation Ib the conditions were pH 5.10, μ 0.0014, and ethanol concentration 5 per cent.

Experiment	Pptn. IIa			Yield	Electrophoretic analysis		
	pH	μ	Ethanol		γ_2 -Globulin	γ_1 -Globulin	β -Globulin
			per cent	gm.	per cent	per cent	per cent
A	7.69	0.045	15	9.6	29	65	6
B	7.46	0.027	15	12.4	31	62	7
C	7.69	0.045	20	14.2	29	68	3

The conditions indicated by Experiment C of Table III give a precipitate consisting largely of γ_1 - and γ_2 -globulins (97 per cent). The ability to separate out such a fraction is considered to be an attainment in itself. This material may be used (1) as a source substance for the preparation of γ_1 -globulin or (2) it may be developed for various clinical uses which may require antibodies present in the γ_1 -globulins of normal human plasma.

The magnitude of this γ_1 - plus γ_2 -globulin fraction in comparison to the γ_2 -protein Fraction II obtained by methods given earlier (6) is shown by Table IV. A very appreciable increase in antibody recovery is indicated by these data.

Separation of γ_1 - and γ_2 -Globulins—Sufficient amounts of starting material were not always available to obtain what might be called high grade globulin pastes for the studies in the development of a satisfactory set of conditions for the separation of γ_1 - and γ_2 -globulins. In the preliminary studies, various precipitates were collected to give a material of the following electrophoretic analysis: 26 per cent γ_2 -, 64 per cent γ_1 -, 7 per cent β -globulin, and 3 per cent albumin. Although this sample contained approximately 10 per cent of impurities (β -globulin and albumin), the presence of these proteins was not considered to be deleterious in such preliminary studies of the actual conditions necessary for satisfactory separation of the γ_1 - and γ_2 -globulins.

TABLE IV
Recovery of γ -Globulin from Fraction II + III

Experiment No.	Dry yield, Fraction II*	Dry yield, fraction γ_1 - + γ_2 -globulin†	Electrophoretic analysis‡		
			γ_2 -Globulin	γ_1 -Globulin	β -Globulin
	gm.	gm.	per cent	per cent	per cent
1	35	28	43	53	4
2	36	26	29	64	7

* From 400 gm. of Fraction II + III paste.

† From residue after extraction of Fraction II from 400 gm. of Fraction II + III paste.

‡ Analysis of γ_1 - + γ_2 -globulin fraction.

In the experiments, 3.5 gm. samples of the protein were suspended in distilled water at 0° to give approximately a 0.3 per cent solution, and 0.05 M acetic acid was added to adjust the solution to the pH range 5.1 to 5.3. After stirring the suspension for from 1 to 12 hours any insoluble material was removed by filtration. The clear solutions were studied to determine the best conditions of ionic strength, pH, ethanol concentration, and temperature, for the separation of γ_1 - and γ_2 -globulins.

A procedure to precipitate the γ_1 -globulin, while maintaining the γ_2 -globulin in solution, appeared to be the most practical means to bring about this separation (Precipitation IIb). The soluble supernatant proteins (largely γ_2 -globulin) were removed by elevating the pH to 7.4 ± 0.1 and bringing the ethanol concentration to 25 per cent.

The effect of pH variations at a concentration of 12 per cent ethanol and an ionic strength of approximately 0.01 at the step Precipitation IIb was first considered. The salts used to adjust the ionic strength were sodium acetate and phosphates. All experiments were evaluated in terms of the amount and purity of γ_1 -globulin separated. The results of some pre-

liminary experiments are shown in Table V. (The amounts of β -globulin and albumin in these preparations are not reported in Tables V to IX, since the emphasis is now on the actual separation of γ_1 - and γ_2 -globulins.)

Increase in pH value resulted in higher yields of γ_1 -globulin with, however, a decreasing ratio of the γ_1 - to γ_2 -globulin. Since both of these considerations were of importance, it was arbitrarily decided to select an intermediate pH range, namely 5.5, for further investigations. With this pH value and 12 per cent ethanol concentration, the effect of ionic strength variation was studied on solutions containing sodium chloride, as well as acetate-phosphate mixtures. The results of such experiments are recorded in Table VI, and suggested that ionic strength in the region 0.005 to 0.015 represented the best conditions for the precipitation of γ_1 -globulin. At higher ionic strengths, increased amounts of γ_2 -globulin were precipitated.

TABLE V
Effect of pH on γ_1 -Globulin Separation

Experiment	Pptn. IIb, 12 per cent ethanol		Yield	Electrophoretic analysis		
	pH	μ		γ_2 -Globulin	γ_1 -Globulin	$\frac{\gamma_1\text{-Globulin}}{\gamma_2\text{-Globulin}}$
			gms.	per cent	per cent	
A	5.25	0.0094	1.12	7	79	11.3
B	5.53	0.0097	1.46	9	72	8.0
C	5.72	0.011	1.81	11	76	6.9

At lower ionic strengths smaller yields were obtained, although the precipitated proteins contained a higher proportion of γ_1 -globulin. For these reasons ionic strengths of 0.010 to 0.015 were used in subsequent experiments. No appreciable differences between yields or purity of product from acetate-phosphate and from sodium chloride systems were observed. In working with small amounts of starting globulin pastes, such as were used in these experiments, considerable variations in the yield of γ_1 -globulin may be experienced. This is evident from the data of Table VI.

A third variable of the system, the concentration of ethanol, was also considered. The results shown in Table VII indicate that at a concentration of 14 per cent ethanol, with pH 5.53 and ionic strength (acetate-phosphate) 0.005, higher yields of γ_1 -globulin were obtained. Other experiments have disclosed that an increase in ionic strength at 14 per cent ethanol will serve to precipitate more γ_2 -globulin. In general, at pH 5.5, the effect of increased ionic strength at a constant ethanol concentration is to salt-out γ_1 -globulin. Such an effect was likewise apparent in the data of Table VI for systems at constant alcohol concentration with variable ionic strength.

As a result of these experiments, the general conditions best suited for the separation of γ_1 - from admixtures with γ_2 -globulin are pH 5.5, ionic strength 0.005 to 0.015, and an ethanol concentration of 10 to 14 per cent. Experiments at the lower ionic strengths were generally carried out with the higher alcohol concentrations and *vice versa*. It is evident that, at the

TABLE VI
Effect of Ionic Strength on γ_1 -Globulin Precipitation

Experiment No.	Pptn. IIb, 12 per cent ethanol		Ionic increment			Yield	Electrophoretic analysis		
	pH	μ	Sodium acetate	Sodium phosphates	NaCl		γ_2 -Globulin	γ_1 -Globulin	$\frac{\gamma_1\text{-Globulin}}{\gamma_2\text{-Globulin}}$
			per cent	per cent	per cent		per cent	per cent	
1-2A	5.49	0.0008	75	25		0.21	No analysis		
1-2B	5.52	0.0052	50	50		1.63	5	83	16.6
1-2C	5.50	0.020	49	51		1.73	9	76	8.4
1-6A	5.48	0.002	32	13	55	0.15	No analysis		
1-6B	5.48	0.0051	12	5	83	1.50	3	90	30.0
1-6C	5.48	0.020	3	1	96	2.00	4	88	22.0
1-7A	5.56	0.006	7	4	89	1.05	3	84	28.0
1-7B	5.56	0.009	5	3	92	1.25	5	84	16.8
1-7C	5.56	0.012	4	2	94	1.32	5	84	16.8
1-7D	5.56	0.015	3	2	95	1.16	5	85	17.0

TABLE VII
Effect of Ethanol Concentration on Precipitation of γ_1 -Globulin

Experiment	Ethanol concentration for Pptn. IIb, pH 5.53, μ 0.005	Yield	Electrophoretic analysis		
			γ_2 -Globulin	γ_1 -Globulin	$\frac{\gamma_1\text{-Globulin}}{\gamma_2\text{-Globulin}}$
	per cent	gm.	per cent	per cent	
A	6	0.80	7	61	8.7
B	10	1.26	5	76	15.2
C	14	1.60	5	79	15.8

penalty of low yield, relatively pure γ_1 -globulins may be obtained, but, if higher yields are desired, the product is contaminated with varying amounts of γ_2 -globulin.

The conditions suggested by the above experiments were applied to a protein sample, 15.7 gm. in weight, which resulted when portions of the proteins obtained in the experiments shown in Table II were pooled. After suspension and treatment as previously indicated, a precipitate (the γ_1 -

globulin) was taken off at pH 5.52, ethanol concentration 10 per cent, and ionic strength 0.006. The supernatant solution was then adjusted to pH 7.47 with Na_2HPO_4 and the ethanol concentration was brought to 20 per

TABLE VIII
Fractional Separation of γ_1 - and γ_2 -Globulin

Material	Weight	Electrophoretic analysis		
		γ_1 -Globulin	γ_2 -Globulin	β -Globulin
	gm.	per cent	per cent	per cent
Starting.....	15.7	30	65	5
pH 5.52 ppt.....	3.8	2.7	94.3	3.0
" 7.47 "	8.9	85	11	4

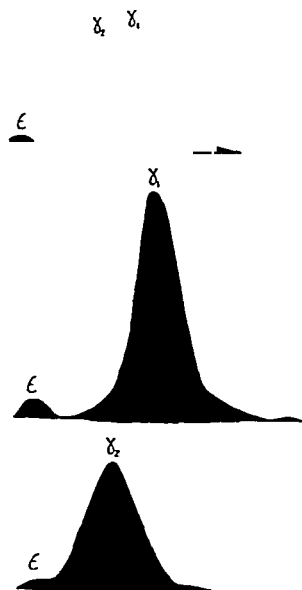


FIG. 4. Electrophoretic diagram of separation of a mixture of γ_1 - and γ_2 -globulins

cent to precipitate the γ_2 -globulin. The results of this experiment are shown in Table VIII. The electrophoretic patterns following this separation are shown in Fig. 4.

Thus, it has been possible to obtain a γ_1 -globulin preparation of relatively high purity. 3 gm. of protein were lost during the fractionation.

However, it can be readily seen that more γ_2 -globulin was recovered in the fractionation than was indicated to be present in the starting material. The difficulty in the electrophoretic resolution and analysis of these two γ -globulins has been previously mentioned.

The precipitates consisting largely of γ_1 -globulin often showed the presence of various amounts of flocculent material after solution in 0.15 M NaCl at physiological pH values. A goodly portion of this insoluble material appears to be the high molecular weight component of the globulin ($s_{20} = 18$ Svedberg units in the ultracentrifuge) which shows marked tendencies to denaturation, as indicated by insolubility. If the γ_1 -globulin paste which had been precipitated at pH 5.5 was suspended in a solution of pH 7.7 and ionic strength approximately 0.05, a portion of the protein failed to go into solution. Removal of this material by centrifugation, followed by filtration of the supernatant solution through a thin pad of filter aid prior to final precipitation of the end-product, gives a protein which has good solution properties. However, portions of the heavier molecular weight constituent have been removed in this process. Studies of the stabilization and properties of this component are in progress.

As a result of this work, the separation and concentration of the proteins of plasma concerned with antibody activity have been extended considerably. Steps in the procedure for the separation of these γ -globulins from the Cohn Fraction II + III (8) of human plasma are summarized in the accompanying outline.

As the description of actual fractionation procedures is completed, it should be remarked that protein pastes (Fraction II + III or a residue thereof) which have undergone relatively long cold storage are more readily fractionated for γ_1 -globulin than are fresh fractions. The basic difference appears to be a greater solubility of the β -globulins in the case of the fresh protein pastes, with a consequently greater difficulty in preparing γ_1 - γ_2 -globulin mixtures which are free of lipoproteins.

By the methods described above, there have been obtained preparations of γ_1 -globulin in excess of 95 per cent purity, as judged by electrophoresis from pastes that have undergone relatively long storage. Sedimentation analysis of the protein shows the following general distribution into light and heavy components: $s_{20w} = 7$ Svedberg units represents approximately 70 per cent, and $s_{20w} = 18$ Svedberg units approximately 30 per cent of the protein. The light component is made up of two subcomponents with sedimentation constants $s_{20w} = 7$ Svedberg units and $s_{20w} = 9$ Svedberg units. The $s_{20w} = 18$ Svedberg units component associated with purified γ_1 -globulin preparations to the extent of 20 to 30 per cent is not found in purified γ_2 -globulin preparations. The other two components, however, have the same sedimentation constant in 1 per cent solution as do the corre-

1 kilo Fraction II + III suspended;
pH 5.1, EtOH 17%, μ 0.01, pro-
tein 1.5-2.5%. Centrifuge

Ppt. Fraction II + III residue; 3000
ml. solution, pH 4.8-5.1 (per kilo);
stir 1-16 hrs.; Na_2HPO_4 to pH 5.1.
Centrifuge

Supernatant; pH 7.0-7.2,
EtOH 25%. Centrifuge

Ppt. γ_2 -globulin

Supernatant
discarded

Ppt. largely β -globulin

Supernatant; 53.3% ethan-
ol to 10% concentration.
Centrifuge

Ppt. largely β -globulin

Supernatant; Na_2HPO_4 to pH
7.7; 53.3% EtOH to 20%

Ppt. largely γ_2 - and γ_1 -globulin. Sus-
pend in pH 5.1-5.3 solution; stir
30-60 min.; Na_2HPO_4 to pH 5.5 ace-
tate-phosphate; μ 0.005-0.015; 53.3%
EtOH to 10-14%. Centrifuge

Supernatant discarded

Ppt. largely γ_1 -globulin. Suspend in
solution; pH 7.7, μ 0.05; stir 30 min.
Centrifuge and filter centrifugate

Supernatant; Na_2HPO_4 to pH
7.2-7.4; 53.3% EtOH to
20%. Centrifuge

Ppt. largely γ_2 -globulin

Supernatant
discarded

Discard ppt.

Supernatant; 53.3% EtOH to
20%. Centrifuge

Ppt. largely γ_1 -globulin

Supernatant discarded

sponding components in γ_2 -globulin preparations. A typical electrophoretic diagram for such a preparation is shown in Fig. 5. In Fig. 6, A and B, are shown the molecular components for the same protein as revealed by ultracentrifuge analysis.

Immunological Studies—The proteins which have been obtained by the new fractionation procedures (Fig. 4 and Table VIII) are of interest be-

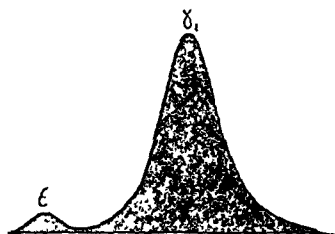


FIG. 5. Electrophoretic diagram of purified γ_1 -globulin

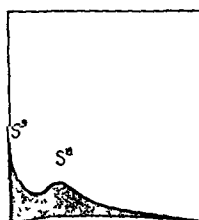


FIG. 6, A

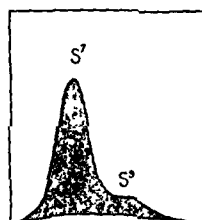


FIG. 6, B

FIG. 6. Sedimentation diagram of purified γ_1 -globulin in 0.15 M sodium chloride at 218,000 times gravity. A, after 15 minutes; B, after 120 minutes.

TABLE IX
Immunological Titers of γ_1 - and γ_2 -Globulins*

Separation No.	γ_2 -Globulin	γ_1 -Globulin	Diphtheria antitoxin	Influenza A		Typhoid agglutinin	
				Hirst	Mouse protection	"H"	"O"
1	95	5	0.4	1.0	0.73	0.5	2.7
	23	72	0.15	0.5	0.3	0.7	15.0
2	97	2	0.87	0.5	1.5	0.2	0.75
	9	70†	0.34	1.0	2.5	0.1	7.5
3	95	5	0.61	1.0	0.7	0.25	1.5
	4	85	2.0	0.5	0.5	0.5	16.0
4‡	0	5	0.004	0.25	0	0.11	1.5

* Titers referred to the standard Fraction A66 of Enders (10).

† This preparation contained 16 per cent albumin.

‡ A preparation containing 87 per cent β -globulin.

cause of their antibody assay values. Some preliminary immunological assays of the γ_1 - and γ_2 -globulins separated from a mixture of these two proteins are shown in Table IX.

A preparation that contained 87 per cent β -globulin and which had been separated from the same starting material as were the γ_1 - and γ_2 -globulins gave very low antibody titers. The latter titers could be explained on the basis of the small amounts of γ -globulins present. Extremely interesting is the association of the major portion of the typhoid "O" agglutinins with the γ_1 -globulin.

The hemagglutinins of plasma are likewise associated with this γ_1 -globulin. A γ_1 - and γ_2 -globulin preparation obtained from a Fraction II + III of pooled plasmas of group O and A (anti- β) individuals by the method of Melin (11) was separated into γ_1 - and γ_2 -globulin fractions. With the test-tube titer hemagglutinin assay method of Pillemer *et al.* (12), it was found that the dilution titer of a 1 per cent solution of a preparation 93 per cent in γ_2 - and 5 per cent in γ_1 -globulin was 1:4 as compared to 1:64 for a preparation containing 73 per cent of γ_1 - and 18 per cent of γ_2 -globulin. An additional γ_2 -globulin preparation obtained from the typed Fraction II + III by our earlier method (6) that was 95 per cent γ_2 - and 2 per cent γ_1 -globulin gave a 1:2 titer when assayed under analogous conditions. The titers of such γ_2 -globulin preparations thus appear to be due to their content of γ_1 -globulin.

The relation of the higher molecular weight components of our γ_2 -globulin preparations to antibody activity has not been determined. Kabat (13) has indicated species differences in the molecular weight of antibodies, the human pneumococcus antibodies being reported to be $s_{20,w} = 7$ Svedberg units in contrast to some other animal species. However, the reports of Deutsch (14) and Davis *et al.* (15) indicate that the Wassermann antibody may be analogous to our heavier molecular component. It is interesting that such molecular species are present in a so called "normal" human serum protein fraction possessing antibody activity, and future studies to elucidate their biological rôles are indicated and are in progress.

DISCUSSION

The γ_1 -globulin appears to be relatively labile in comparison with γ_2 -globulin, as judged by stability to heat. However, it has been demonstrated that it carries certain of the antibody proteins. This protein has sometimes been called β_2 -globulin and is in many respects analogous to the "T" component of horse hyperimmune sera (4). Since the γ_1 -globulin prepared in this work, from residues that had undergone long standing at low temperature, is free of lipide and cholesterol and has associated with it a variety of antibodies, it is biologically and physically in a category with normal serum γ -globulin. Because of the association of the term β -globulin with the lipoproteins of plasma, we feel that the term γ_1 -globulin is to be

preferred, and it has been used in this report. Thus, by analogy, the normal serum γ -globulin is to be designated as γ_2 -globulin. Such a description will be less confusing in relating the antibody function of plasma proteins to a similar and easily recognizable terminology that has already established the precedent of association of the term γ -globulin with antibody function.

Immunologically active proteins similar to the γ_1 - and γ_2 -globulins from human plasma apparently have been prepared from bovine serum by Smith (16). As already mentioned, similar proteins are present in the serum of the horse (4).

The γ_1 -globulin appears to comprise approximately 2 to 3 per cent of the normal human plasma proteins. By the method described above, approximately 50 to 70 per cent of this protein is obtained in mixture with γ_2 -globulin to give a new human plasma protein fraction. While this fraction as a whole has not been investigated clinically, immunological assay data indicate a significant increase in antibody yield. The usual recovery of this new γ_1 - plus γ_2 -globulin fraction from a kilo of the Fraction II + III paste is approximately 60 gm. of dry protein. This mixture can be separated into its component proteins if desired, with some fractionation losses. This brings the over-all yield of γ_2 -globulin to approximately 95 per cent if our method for preparation of γ_2 -globulin, described in Paper III of this series (6), is first applied to Fraction II + III. The exact yields in the separation of γ_1 - and γ_2 -globulin mixtures eventually will be evaluated in terms of electrophoretic analysis with known mixtures of γ_1 - and γ_2 -globulins.

Detailed biological and physical properties of the purified γ_1 -globulin will be described in future reports.

The raw materials in the form of by-product pastes containing β - and γ -globulins (obtained in the processing of normal human plasma for albumin) were supplied through the generous cooperation of Dr. F. A. Eberly of The Upjohn Company, Dr. J. D. Porsche of Armour and Company, and Dr. F. F. Johnson of the Cutter Laboratories.

The typed Fraction II + III was obtained through the courtesy of the Department of Physical Chemistry, Harvard Medical School.

All immunological assays other than hemagglutinin titers were carried out by Dr. J. F. Enders and Miss J. C. Sullivan of the Harvard Medical School. We wish to thank these individuals for their generous aid and interest.

We wish gratefully to acknowledge the helpful suggestions of Dr. J. W. Williams during the course of this work.

SUMMARY

As a result of investigations having to do with the separation of the globulin constituents of normal human plasma, procedures have been developed for the isolation of a fraction consisting of two proteins concerned with antibody function. The starting material is a by-product protein paste from which some normal serum γ -globulin has been previously extracted. From this new fraction it has been possible to separate a protein which we have designated as γ_1 -globulin to differentiate it from the other component, the usual γ_2 -globulin of normal serum.

The application of these procedures is also important in that a considerable increase in yield of the proteins of human plasma concerned with antibody production results. Thus, the yield of γ_2 -globulin which had been already increased from some 50 to 80 per cent by our earlier investigations now is brought to the over-all figure of 95 per cent by the successive application of the prior and the new conditions in the subfractionation of Fraction II + III. The γ_1 -globulin is also an interesting protein from the point of view of its antibody content.

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PAIRED FEEDING IN THE STUDY OF THE COUNTERACTION BY NICOTINIC ACID AND TRYPTOPHANE OF THE GROWTH-DEPRESSING EFFECT OF CORN IN RATS

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The possibility that a deficiency of certain essential amino acids might be a factor in the etiology of pellagra was postulated as early as 1914 by Voegtlin (1). Chick and Hume (2) reported in 1920 the production in monkeys of symptoms closely resembling those of pellagra by prolonged feeding on a diet of low protein content. The administration of tryptophane to one monkey staved off death for many weeks. Some beneficial results from the treatment of pellagrins with tryptophane and cystine were also observed by Goldberger and Tanner (3). Chick, however, rejected the theories that pellagra is due to an amino acid deficiency or to vitamin B₂ deficiency because they did not explain the association of epidemic pellagra with maize consumption. She proposed as a theory, "Pellagra is caused by a toxic substance derived from the maize diet, which can be corrected by sufficient 'good' protein, or perhaps by sufficient vitamin B₂" (4).

Elvehjem and his coworkers (5) demonstrated the specific anti-black-tongue activity of nicotinic acid and nicotinamide. Very shortly after, nicotinic acid was also successfully used in human pellagra. The work of Dann (6) and of others has shown that a dietary source of nicotinic acid is not required by the rat. Recently Krehl, Teply, Sarma, and Elvehjem (7, 8) reported a growth inhibition in rats, attributable to nicotinic acid deficiency, by adding corn or corn grits to a purified diet very low in nicotinic acid. This growth retardation could be prevented by tryptophane as well as by nicotinic acid or by an increase in the level of casein.

It is interesting in this connection that Szymanska and Funk (9) attributed an appetite-stimulating and weight-preserving action to nicotinic acid and the amide. Later, Funk and Funk (10) also reported that nicotinic acid, and especially nicotinamide, produced in rats and pigeons kept on a diet free of the vitamin B₂ complex, a much larger food intake and better weight compared with the controls. This suggested to György (11) the supplementation of his vitamin B₂-deficient diets with nicotinic acid. No difference was observed in the production or progress of the rat acrodynia and in the rate of growth in the experiments. However, "the addition of nicotinic acid to the diet surprisingly resulted in the complete suppression of panmyelophthisis, a complication which was a common occur-

rence in rats kept under the same nutritional conditions but without nicotinic acid."

More recently Wooley and Sebrell (12) found that young rabbits fed a purified ration supplemented with pure vitamin preparations including nicotinic acid grew at a nearly normal rate. Rabbits receiving no nicotinic acid lost weight and died. However, rabbits fed the diet containing an adequate amount of nicotinic acid, but no more food than their litter mates receiving the nicotinic acid-deficient ration, died as early as the deficient animals and showed similar symptoms. The authors stated, "It is possible that the signs and symptoms shown by these rabbits were due to slow starvation brought about by anorexia when nicotinic acid was excluded from the diet."

The possibility that a depression of appetite may account for the effect of corn in depressing growth and its counteraction by nicotinic acid or tryptophane is an obvious suggestion of the literature cited. The question

TABLE I
Results of ad Libitum Feeding Trial

Ration	Mean food intake	Mean gain in weight	Standard error of mean gain in weight
	gm. per wk.	gm. per wk.	gm. per wk.
Basal.....	71	25.5	1.0
" + 40% corn grits.....	45	8.8	1.4
" + 40% " " + 1.5 mg. % nicotinic acid.....	80	25.3	1.6
Basal + 40% corn grits + 0.1 % l(-)-tryptophane.....	78	24.4	1.8

arises, therefore, to what extent is the growth retardation produced due to a specific vitamin (or amino acid) requirement, and to what extent is it due merely to generalized undernutrition. The distinction between a generalized undernutrition and a deficiency of nicotinic acid (or tryptophane) would be revealed by the use of paired feeding to equalize the food intakes of comparative animals.

EXPERIMENTAL

In the experiments here reported the conditions described by the Wisconsin group were essentially duplicated, employing a paired feeding technique as well as *ad libitum* feeding. The basal ration used had the following composition: Labco casein (three times extracted with 95 per cent ethanol) 15, sucrose 78, corn oil 3, Salts 4 (13) 4, and cystine 0.15 parts. Vitamins were incorporated in the ration at the following levels: thiamine

TABLE II
Results of Paired Feeding Trials

Rat No.	Supplement to basal ration	Food intake	Mean gain in weight	Difference in gain	Probability of chance outcome*
		gm. per wk.	gm. per wk.	gm. per wk.	
1	None	34	8.2	3.2	
2	40% corn grits	34	5.0		
3	None	39	10.4	2.3	
4	40% corn grits	39	8.1		
5	None	45	15.6	2.8	
6	40% corn grits	45	12.8		
7	None	41	11.2	1.3	
8	40% corn grits	41	9.9		
9	None	38	12.5	3.5	
10	40% corn grits	38	9.0		
Means	None	39	11.6	2.6	<0.0019
	40% corn grits	39	9.0		
11	40% corn grits	32	5.0		
12	Same + nicotinic acid	32	6.4	1.4	
13	40% corn grits	35	7.5		
14	Same + nicotinic acid	35	8.8	1.3	
15	40% corn grits	38	8.2		
16	Same + nicotinic acid	38	9.2	1.0	
17	40% corn grits	43	8.2		
18	Same + nicotinic acid	43	9.4	1.2	
19	40% corn grits	37	7.8		
20	Same + nicotinic acid	37	8.8	1.0	
Means	40% corn grits	37	7.3		<0.0019
	Same + nicotinic acid	37	8.5	1.2	
21	40% corn grits	50	13.5		
22	Same + tryptophane	50	14.6	1.1	
23	40% corn grits	33	6.8		
24	Same + tryptophane	33	7.2	0.4	
25	40% corn grits	36	7.4		
26	Same + tryptophane	36	8.5	1.1	
27	40% corn grits	37	5.8		
28	Same + tryptophane	37	8.0	2.2	
29	40% corn grits	37	9.0		
30	Same + tryptophane	37	9.0	0	
Means	40% corn grits	39	8.5		0.031
	Same + tryptophane	39	9.5	1.0	

* "Student," *Biometrika*, 6, 1 (1908).

0.2, riboflavin 0.3, pyridoxine 0.25, calcium pantothenate 2.0, choline chloride 100, inositol 10, 2-methylnaphthoquinone 0.1, and biotin 0.01 mg. per 100 gm., respectively. Halibut liver oil (diluted 1:2 with corn oil) was fed at a level of 2 drops per week, with α -tocopherol included at 0.5 mg. per drop. Synthetic folic acid¹ was used in place of the norit eluate preparation at a level of 11.5 γ per 100 gm. of ration. The vitamin and cystine levels were maintained in both the basal and corn-supplemented rations and the nicotinic acid content of the basal ration was 0.003 mg. per 100 gm. by assay with *Lactobacillus arabinosus*.

Fifty weanling male litter mate rats ranging in weight from 43 to 48 gm. were used in the investigation. Five pairs of rats were employed in each of three feeding tests involving equalized feeding and five rats in each of four groups in the *ad libitum* feeding tests. A record was kept of the food consumption in the *ad libitum* feeding. The experiments were all of 5 weeks duration. The results are summarized in Tables I and II.

The *ad libitum* feeding tests reveal that the addition of corn grits to the basal ration results in a marked depression of growth associated with a decreased food consumption. The addition of nicotinic acid or of tryptophane to the corn-supplemented ration produced a marked improvement in food consumption and a gain in weight corresponding to that obtained on the basal ration alone. The paired feeding tests demonstrate that the incorporation of nicotinic acid or tryptophane in the corn-supplemented ration produced slight but statistically significant greater gains in young rats at the same level of food consumption. Thus under the conditions of these experiments both nicotinic acid and tryptophane produced a somewhat more efficient use of the ration for growth.

In a report which appeared in print after these experiments were initiated Krehl *et al.* (14) observed that the animals fed the corn-supplemented ration developed a rough fur coat with occasional alopecia and an accumulation of a reddish exudate of porphyrin-like material, as originally described by Chick, Macrae, and Worden (15), in animals deprived of the filtrate-fraction, on the paws, around the nose, and on the tail. These symptoms also developed in our experiments among those rats receiving the basal ration plus corn grits, and were prevented by either nicotinic acid or tryptophane both in the *ad libitum* tests and in the paired feeding tests.

DISCUSSION

The paired feeding tests demonstrate that most of the marked stimulation in growth produced by the nicotinic acid or tryptophane supplements to the corn grits ration was associated with an increased caloric intake.

¹ We wish to thank Dr. E. L. R. Stokstad of the Lederle Laboratories, Inc., Pearl River, New York, for kindly supplying us with synthetic folic acid.

When the caloric intakes were equalized the growth stimulation of these supplements was reduced from 16 gm. per week to an average of 1.0 to 1.2 gm. Even the depression in growth induced by the addition of 40 per cent corn grits to the basal ration largely disappears when the rations are compared on the equicaloric basis, decreasing from an average of 16.7 gm. gain per week to one of 2.6 gm. The latter figures may be taken to represent the inherent effect of the dietary modifications imposed, uncomplicated by associated effects upon appetite. But the reality of these differences in inherent growth-promoting value, however small, seems established by the paired feeding tests, though no data are available to judge to what extent these differences may be due merely to differences in water and fat contents of the carcasses of the test animals.

Taking at their face value these stimulations in growth on equicaloric intakes of food induced by the nicotinic acid or tryptophane supplement to the corn grits diet, it seems unnecessary to attribute them to changes induced in the synthetic activities of the microorganisms of the intestinal tract, since in *ad libitum* feeding tests Krehl and associates (14) secured similar results with nicotinic acid and tryptophane supplements whether administered to the experimental rats by mouth or subcutaneously. They can more reasonably be associated with functions outside the sphere of tissue protein synthesis. Individual amino acids are precursors of hormones and in other guises may enter into reactions in which vitamins also participate. A case in point is the association of methionine and choline in transmethylation reactions and in preserving normal hepatorenal functioning. Either methionine or choline may rectify the deleterious effects of a low level of dietary protein containing a low concentration of methionine. This analogy suggests a similar relationship between nicotinic acid and tryptophane when the dietary intake of both is low. Under these conditions the ability of the rat to synthesize nicotinic acid may be inadequate to meet the situation, just as its ability to synthesize choline may be inadequate when the methionine intake is low. Tryptophane may substitute for nicotinic acid, just as methionine may substitute for choline. In fact, in the pig, an animal whose ability to synthesize nicotinic acid is absent or negligible, a high protein diet may largely obviate the need for a dietary source of nicotinic acid, conceivably because of the excess tryptophane thus provided (16). The biological function, whatever it may prove to be, in which nicotinic acid and tryptophane may participate interchangeably, seems to be associated with the maintenance of a normal, healthy alimentary tract upon which a normal appetite depends.

Rosen, Huff, and Perlzweig (17) in a recent note reported a drop in urinary excretion of nicotinic acid by adult rats when casein was replaced by gelatin, and an increase in nicotinic acid excretion on addition of 50

mg. of tryptophane either orally or subcutaneously. They interpret these observations to indicate that tryptophane may be the important precursor of nicotinic acid synthesis in the rat. These data may also be interpreted as indicating a sparing action of nicotinic acid by tryptophane. Singal *et al.* (18) found a sharp decrease in the urinary nicotinic acid of rats placed upon the low protein, corn grits diet of Krehl *et al.* (7). The addition of 1.0 mg. per cent of nicotinic acid or the increase of the casein content of the diet to 20 per cent (which counteracts the growth depression induced by addition of corn) did not alter the amount of nicotinic acid excreted. When 0.5 per cent *l* (-)-tryptophane was included in the diet, a large increase was observed in urinary nicotinic acid which, upon acid hydrolysis but not alkaline hydrolysis, was increased further some 2- to 3-fold.

Briggs (19) has reported that the addition of 10 per cent gelatin to a diet which contains 18 per cent casein caused a marked depression in the growth rate of chicks and caused the production of typical nicotinic acid deficiency symptoms. All inhibitory action was counteracted by supplementing with either 5 mg. of nicotinic acid or with 200 mg. of *dl*-tryptophane per 100 gm. of diet. In a previous report, Briggs and other (20) found that chicks receiving the basal ration synthesized only about one-sixth of their total nicotinic acid requirement. Feeding of succinyl sulfathiazole revealed that little or no intestinal synthesis of nicotinic acid occurs in chicks receiving a purified diet.

SUMMARY

The addition of as much as 40 per cent of corn grits to a basal diet low in nicotinic acid and containing 15 per cent of casein depresses the food consumption of growing rats and the rate of growth. The depression of growth is largely, but not entirely, due to the depression of the desire for food, since it still occurs when the caloric intake of the animals on the basal diet alone is restricted to that of the animals on the corn grits ration.

The addition of either nicotinic acid or of tryptophane to the corn grits ration induces a resumption of growth in the rats consuming it, largely, but not entirely, associated with a concomitant increase in food consumption.

It is concluded from these and other observations reported in the literature that the inherent physiological effects of nicotinic acid and tryptophane, not dependent upon an increase in the intake of food, are probably not related to a modification in the synthetic capacities of the body or of the microorganisms of the intestinal tract, but possibly to some unknown function in the body in which nicotinic acid and tryptophane participate interchangeably, analogous to the interchangeable relations in metabolism of choline and methionine.

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THE OXIDATION OF CATECHOL BY TYROSINASE

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Several investigators (1-5) have concluded that the complete oxidation of catechol in the presence of tyrosinase requires only 2 atoms of oxygen per molecule. We have reinvestigated this enzymic reaction and have found the consumption of oxygen to be dependent upon the conditions of the experiment. Under some circumstances, it may be as high as 3 atoms per molecule.

EXPERIMENTAL

The oxygen consumption measurements were made with Barcroft differential type manometers fitted with side arm flasks of approximately 18 ml. capacity. The total volume of fluid in each flask was always brought to 3.0 ml. by varying the quantity of buffer solution added to the main compartment of the flask. The tyrosinase was diluted with water until 0.5 ml. contained the desired concentration of the enzyme and this quantity was then delivered into the side arm of the flask. After introduction of the solutions, the manometer flasks were immersed in a water bath at 25.6° and flushed out with water-saturated oxygen for 5 minutes while shaking at a rate of 110 oscillations per minute. After closing the manometers, 5 minutes were allowed to assure equilibrium. The enzyme was then tipped from the side arm and the measurement of the rate of the reaction begun.

The volumes of the flasks and manometer tubes were determined from the weight of mercury required to fill them and the conversion factors for O_2 and CO_2 calculated according to Dixon (6). As a further check the CO_2 factors were determined by tipping an excess of H_2SO_4 on a known amount of $NaHCO_3$. The greatest difference between any pair of the CO_2 factors determined by the two methods was less than 2 per cent. The 1.0 ml. pipette used to measure the catechol solutions delivered with an error of less than 0.5 per cent.

Two tyrosinase preparations were employed in this study. The first (enzyme I) was made by extracting the natural substrate from frozen, ground mushrooms (7). The subsequent steps followed were those described by Ludwig and Nelson (4) to the point at which our preparation

corresponded to their Preparation C101A. It initially contained 311 catecholase units per ml., or 104 units per mg. of dry weight, as determined by the chronometric method of Miller *et al.* (7). At the time it was employed in this study some months later, it contained 273 catecholase units per ml., or 90 units per mg. of dry weight. Enzyme II was obtained by following the directions of Ludwig and Nelson (4) throughout and again corresponded to their Preparation C101A. It contained 96.5 catecholase units per ml., or 121 units per mg. of dry weight. This enzyme was used within a few days of its preparation.

The catechol was obtained from the Eastman Kodak Company, and melted at 104–105° (corrected).

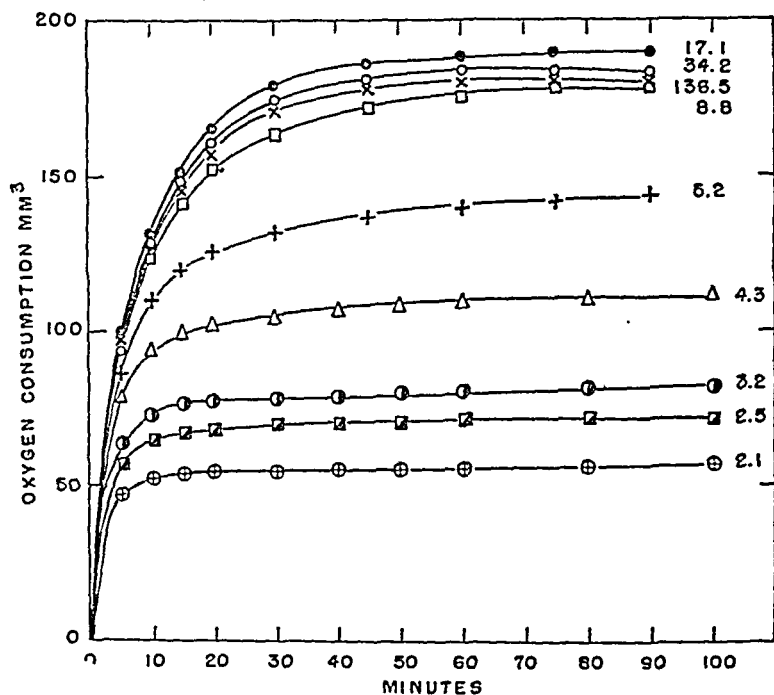


FIG. 1. The oxygen consumed during the enzymic oxidation of 0.73 mg. of catechol with increasing quantities of tyrosinase. The number of catecholase units is indicated for each curve. McIlvain's phosphate-citrate buffer was used (pH 5.1).

Results

The measurement of the oxygen consumption during the enzymic oxidation of catechol was carried out in a series of experiments in which enzyme concentration, catechol concentration, and pH were systematically varied.

Fig. 1 shows the results obtained when the enzyme concentration was varied from 2.1 to 137 catecholase units per 3 ml., while the amount of catechol was fixed at 0.73 mg. and the pH at 5.1. The total oxygen consumed increased from 57 c.mm. with 2.1 enzyme units to 188 c.mm. with

17 units. With 137 units, 180 c.mm. were consumed. The volumes of oxygen utilized at each enzyme concentration, as shown in Fig. 1, were recalculated in terms of atoms of oxygen consumed per molecule of catechol. The data so obtained, up to 34 units of enzyme, are plotted in Fig. 2. The atoms of oxygen consumed increased rapidly to 2.5 with approximately 10 units of enzyme. Further increase in the concentration of enzyme resulted in no further increase in the oxygen taken up.¹

The results obtained with fixed enzyme concentration and varying concentrations of catechol at different hydrogen ion concentrations are

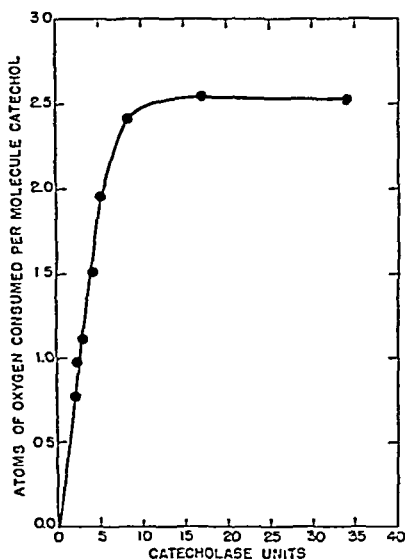


FIG. 2. Atoms of oxygen consumed per molecule of catechol as a function of catecholase units in 3 ml. of reaction volume.

shown in Figs. 3, 4, and 5. The enzyme concentration was fixed in these experiments at 41 units because at this value it was unlikely that the total oxygen consumed would be a critical function of the enzyme concentration (Fig. 2). The upper graph in Fig. 3 shows the results obtained at pH 3.1. Only 0.36 atom of oxygen was consumed per molecule of catechol oxidized, but the enzyme was found to be rapidly inactivated at this hydrogen ion

¹ After this manuscript was completed, a sample of tyrosinase was kindly furnished us by Dr. Irwin Sizer. This sample was described as containing 2750 chronometric catecholase units per ml., or per 2.3 mg. of extractive solids. The experiments described in Figs. 1 and 2 were repeated with this preparation. The number of atoms of oxygen consumed per molecule of catechol reached 2.53 at 34 units of enzyme. Higher concentrations of enzyme gave the same value.

concentration. This inactivation was proved by the addition of more catechol from the second side arm of the manometer flask without further consumption of oxygen. At each of the other hydrogen ion concentrations the enzyme was shown to be active at the conclusion of the experiments. The lower graph in Fig. 3 shows the results obtained at pH 4.5. The

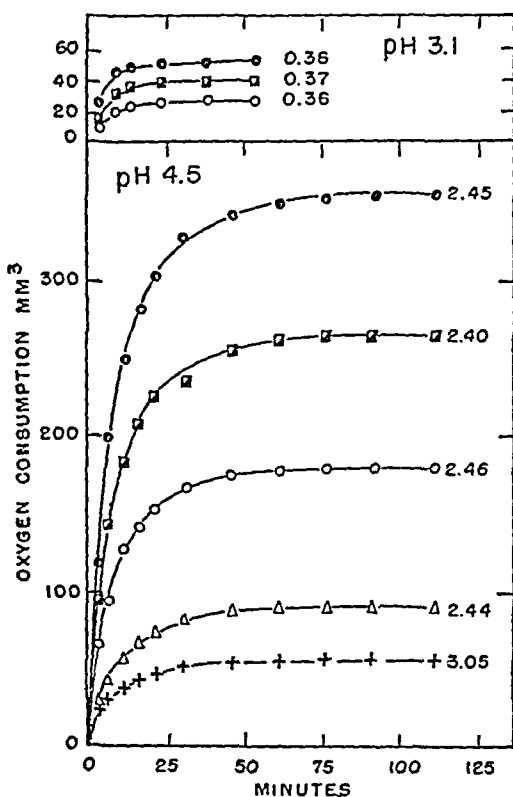


FIG. 3. The oxygen consumed in the oxidation of increasing concentrations of catechol at pH 3.1 and 4.5, with enzyme concentration fixed at 41 catecholase units. Reading from the lowest curve up, the amounts of catechol used per 3 ml. of reaction volume were 0.18 mg., 0.37 mg., 0.73 mg., 1.10 mg., and 1.46 mg. McIlvain's phosphate-citrate buffer was used. The atoms of oxygen consumed per molecule of catechol are indicated for each curve.

number of atoms of oxygen consumed per molecule of catechol varied between 2.45 and 3.05. The highest value was obtained with the lowest concentration of catechol.

In order to prove that the number of atoms of oxygen consumed was not a characteristic peculiar to the enzyme initially employed, two preparations were used to obtain the data illustrated in Fig. 4. The data given

in the left half of the figure were obtained with enzyme I. The atoms of oxygen consumed per molecule of catechol varied between 2.46 and 2.88. In the right half of the same figure are the data obtained with enzyme II. The atoms of oxygen consumed per molecule of catechol varied in this case between 2.47 and 2.80. The experimental conditions for these two sets of data were the same, except for the enzyme preparation, and the results were identical.

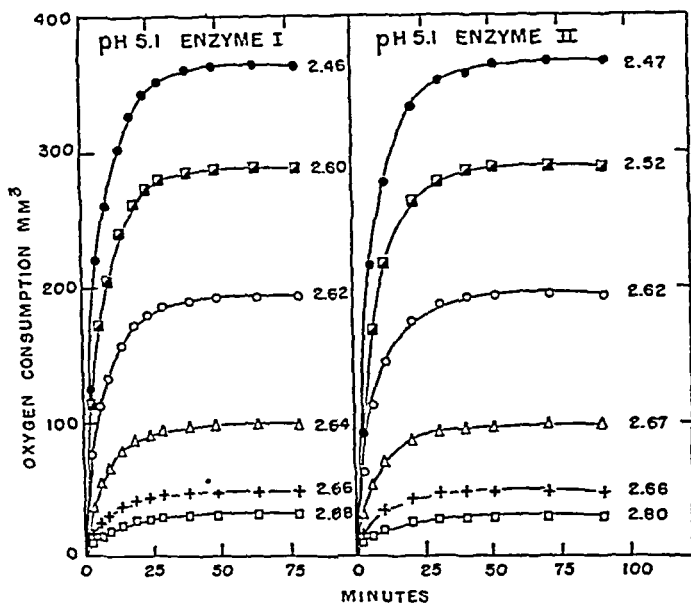


FIG. 4. The oxygen consumed in the oxidation of increasing concentrations of catechol by two enzyme preparations at pH 5.1 (McIlvain's buffer). The concentration of enzyme I was 41 catecholase units per 3 ml. and the concentration of enzyme II was 39 catecholase units per 3 ml. Reading from the lowest curve up, the amounts of catechol per 3 ml. were 0.11, 0.18, 0.37, 0.73, 1.10, and 1.46 mg.

Fig. 5 shows the results obtained at pH 7.0 and at pH 7.9. It was found necessary at these hydrogen ion concentrations to extend the duration of the measurements because a slow but appreciable oxidation proceeded for hours after the initial rapid reaction. At pH 7.0, the atoms of oxygen consumed per molecule of catechol at 46 minutes varied between 1.98 and 2.37; at 30 hours these values varied between 2.35 and 3.35. At pH 7.9 the atoms of oxygen consumed at 55 minutes varied between 1.97 and 2.34; at 36 hours these values then varied between 2.34 and 3.01.

Again, the highest consumption of oxygen per molecule of catechol was obtained with the lowest concentrations of catechol.

The experiments reported above were controlled by adding 41 units of enzyme to the buffer (pH 7.0) in the absence of catechol. No significant oxygen consumption was observed over a period of 7 hours. As a further control, to obviate the possibility of the oxidation of citrate, an experiment

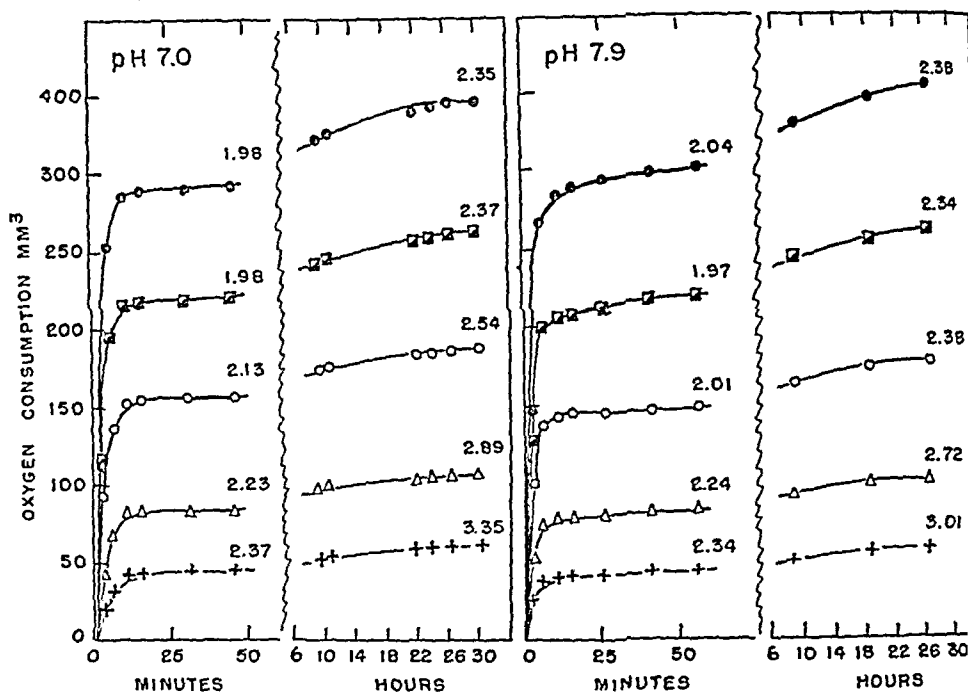


Fig. 5. The oxygen consumed in the oxidation of increasing concentrations of catechol at pH 7.0 and at 7.9 (McIlvain's buffer), with enzyme concentration fixed at 41 catecholase units per 3 ml. Reading from the lowest curve up, the amounts of catechol per 3 ml. were 0.18 mg., 0.37 mg., 0.73 mg., 1.10 mg., and 1.46 mg. The atoms of oxygen consumed per molecule of catechol are indicated at approximately 50 minutes and again at the termination of the experiment.

was carried out with phosphate buffer at pH 5.1. The results of this experiment and a simultaneous experiment with citrate-phosphate buffer at the same pH are shown in Fig. 6. While the kinetics of the two reactions differed, the numbered atoms of oxygen consumed per molecule of catechol were approximately the same at 145 minutes.

DISCUSSION

Fig. 2 shows that with fixed catechol concentration (0.73 mg. per 3 ml.) the amount of oxygen consumed increases with the amount of enzyme used,

up to 2.5 atoms with 10 units. Beyond 10 units there is no further increase in oxygen utilization. The first part of this curve corroborates Graubard and Nelson (8, 9) who showed that, within limits, by increasing the amount of enzyme a greater quantity of oxygen was consumed. The results they obtained did not, however, reach 2.0 atoms of oxygen per molecule of catechol. Robinson and McCance (1) in a single experiment showed that under the conditions employed 2 atoms of oxygen were consumed per molecule. The same value was later obtained by Wagreich and Nelson (2), Dawson and Nelson (3), Ludwig and Nelson (4), and Kubowitz (5). However, none of these authors published a systematic study of the effect of varying enzyme and catechol concentration upon the total consumption of oxygen.

The low consumptions of oxygen at pH 3.1 (Fig. 3) are the results of the inactivation of the enzyme and confirm the findings of Graubard and Nelson (8).

By comparing the results in Figs. 3, 4, and 5 it may be noted that as the pH was increased the initial rate of oxidation was increased, but the time required for complete oxidation was increased. It seems therefore that, of the reactions involved in the complete oxidation of catechol by tyrosinase, at least one is accelerated by increasing the concentration of hydroxyl ions, while another is accelerated by increasing the concentration of hydrogen ions. Furthermore, these reactions are rate-determining steps in the over-all oxidation.

From the results as a whole, it may be seen that two conditions lead to high consumption of oxygen per molecule of catechol. These are high enzyme concentration and low catechol concentration. Figs. 3, 4, and 5 show that the highest oxygen consumptions per molecule of catechol were found when the lowest concentrations of catechol were used. It may be pointed out that our ratios of enzyme to substrate concentration were, in every instance of complete oxidation, greater than in any previously reported. Some other possibilities may be advanced to explain our relatively high values for oxygen consumption. One is that some oxidizable substance has been added with the enzyme. This, however, seems to be precluded by the fact that the addition of 13 times as much enzyme as is required for the consumption of 2.5 atoms of oxygen per molecule of catechol does not further increase this value (Fig. 1). Another possibility is that the citrate of the buffer was oxidized by the tyrosinase-catechol system. This also seems unlikely because the enzymic oxidation of catechol in the absence of citrate (Fig. 6) resulted in the consumption of 2.5 atoms of oxygen. Furthermore, the addition of 41 units of enzyme to phosphate-citrate buffer in the absence of catechol resulted in no significant consumption of oxygen.

Fig. 6 illustrates that the rates of the reaction in the two buffer systems were different. The rate of oxidation in phosphate buffer changed sharply at a consumption of approximately 1 atom per molecule of catechol. A similar but much more pronounced change has been observed by Dawson and Nelson (3) and interpreted by them (10) as due to a slow hydration

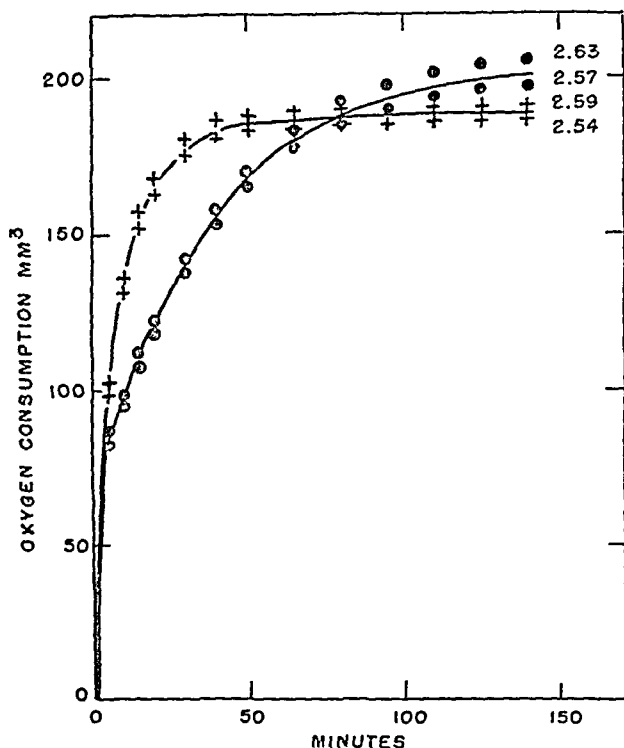


FIG. 6. The effect of type of buffer on the rate and total oxygen consumption in the enzymic oxidation of catechol at pH 5.1. In the experiment indicated by + McIlvain's buffer and 0.72 mg. of catechol were used with 14 catecholase units; in the experiment indicated by ● the buffer was 0.2 M NaH_2PO_4 - Na_2HPO_4 , and 0.75 mg. of catechol was used with 14 catecholase units of enzyme. The atoms of oxygen consumed per molecule of catechol are indicated for each curve.

of *o*-benzoquinone at pH 5 in dilute solutions. Since only the nature of the buffer was changed in the experiments illustrated in Fig. 6, it is evident that a change of rate at 1 atom may also be produced by a change in the buffer system.

The fact that more than 2 atoms of oxygen were consumed during the complete enzymic oxidation of catechol, together with the failure to detect hydroxy-*p*-quinone spectroscopically during this oxidation (11), is not

consistent with the mechanism of oxidation proposed by Wagreich and Nelson (2). We have also determined the oxygen consumed during the enzymic oxidation of hydroxyhydroquinone and have found that 2 atoms are utilized per molecule. These results, together with others bearing upon the mechanism of the enzymic oxidation of catechol and hydroxyhydroquinone, will be published in subsequent papers.

SUMMARY

1. The oxygen consumed during the oxidation of 0.73 mg. of catechol in the presence of tyrosinase was dependent upon the concentration of enzyme, only up to 3.3 catecholase units per ml. Further increase in enzyme concentration produced no further increase in oxygen consumption.

2. The number of atoms of oxygen consumed during the complete oxidation of a molecule of catechol in the presence of tyrosinase was dependent upon the concentration of catechol, being highest at the lowest concentration.

3. The kinetics of the oxidation of catechol in the presence of tyrosinase is dependent upon the hydrogen ion concentration and upon the nature of the buffer.

4. Under the conditions of pH, catechol concentration, and enzyme concentration employed, the values for the number of atoms of oxygen consumed during the complete enzymic oxidation of a molecule of catechol varied between 2.34 and 3.35.

We wish to thank Anne H. Wright for technical assistance in the conduct of the manometric measurements.

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THE VITAMIN B₆ GROUP

VIII. BIOLOGICAL ASSAY OF PYRIDOXAL, PYRIDOXAMINE, AND PYRIDOXINE*

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Though various chemical and microbiological methods have been developed for the estimation of vitamin B₆ in whole (2-4) or in part (5), animal assays are still necessary for testing the accuracy of these new methods as they apply to animal nutrition. It is also important that the relative potency of pyridoxine, pyridoxamine, and pyridoxal for animals be known. Two different purified rations have been used most frequently for the rat assay. The one devised by Dimick and Schreier (6), besides containing the usual purified constituents of a basal ration, also contains beef liver extract, while the one employed by Conger and Elvehjem (7) contains a fullers' earth filtrate of a butanol extract of 1:20 liver powder. In either case, the crude extracts contribute a definite but variable amount of vitamin B₆ to the basal ration and hence their use constitutes a distinct disadvantage. Further, neither of these two rations allows maximum growth in rats when supplemented with large amounts of vitamin B₆. Clarke and Lechychka (8) have described a biological assay for vitamin B₆ in which rats were kept on the deficient diet for a period of 4 weeks or more before feeding supplements containing the vitamin. Since the newer members of the vitamin B complex like biotin, inositol, and synthetic folic acid are now available, the present investigation was initiated with the object of devising a vitamin B₆-free ration which would permit the minimum possible growth of rats on the deficient diet and the maximum growth when optimum amounts of vitamin B₆ were added.

EXPERIMENTAL

The basal ration originally employed for the production of vitamin B₆ deficiency in rats consisted of carbohydrate 75 per cent, protein 18 per cent,

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corn oil 3 per cent, Salts 4 (9) 4 per cent, and optimum amounts of thiamine, riboflavin, calcium pantothenate, choline chloride, 2-methyl-1,4-naphthoquinone, inositol, nicotinic acid, and biotin. Male weanling rats (Sprague-Dawley) 21 days old, weighing 35 to 40 gm., were employed for these experiments. At least three animals were placed in each group and experiments on many groups were repeated several times in order to confirm the results. The values given in Tables I to V represent the average weight gain in gm. per week for the three animals of each group over a period of 4 weeks.

Several different carbohydrates and proteins were tried with the object of devising a ration which would permit the minimum growth gain in the absence of vitamin B₆. The results are given in Table I. When the diet

TABLE I
Growth of Rats on Various Vitamin B₆-Deficient Diets

Diet No.	Carbohydrate	Protein	Weight gain per wk. and range; average of 4 wks. growth
			gm.
1	Sucrose	18% casein (Smaco)	9 (6-11)
2	"	18% blood fibrin	5 (4- 5)
3	"	9% casein (Labco) + 9% blood fibrin	6 (5- 7)
4	"	10% egg albumin + 10% casein (Smaco)	10 (9-12)
5	Dextrin	18% casein (Smaco)	23 (16-28)
6	"	18% fibrin	18 (16-19)
7	Glucose	18% casein (Smaco)	9 (7-11)
8	Dextrin	18% " " + 0.5% sulfathalidine 0.025 mg.% synthetic folic acid	10 (6-16)
9	Same as (8) + 50 γ pyridoxine HCl per 100 gm. ration		23 (22-23)

contained sucrose as the carbohydrate and casein (Smaco) as the protein, the rats grew 9 gm. per week. When blood fibrin was substituted for casein the rate of gain was reduced to 5 gm. per week. This difference in growth was greater than could be accounted for by the fact that casein (Smaco) at 18 per cent level contributed 4.7 γ of vitamin B₆ per 100 gm. of ration, as compared to only 1.0 γ for blood fibrin as determined by the yeast growth method (3). The results with glucose were similar to those with sucrose, but when dextrin was used a remarkable increase in growth resulted, even though the dextrin by yeast assay contained only 0.04 γ per gm. of vitamin B₆. Dextrin is known to stimulate intestinal synthesis (10, 11) and it was therefore thought that with dextrin as the carbohydrate the intestinal flora produced more vitamin B₆ which was subsequently utilized by the rat. Several experiments indicated that this is a plausible

explanation. Sulfathalidine, which is known to inhibit the growth of certain intestinal microorganisms, was added to the dextrin ration at 0.5 per cent level together with biotin and synthetic folic acid. The rate of growth was markedly decreased. The addition of 50 γ of pyridoxine hydrochloride per 100 gm. of ration restored the rate of growth to the original level.

Results from analysis of the urine excreted by rats maintained on sucrose and dextrin rations also confirmed the above observations. Lepkovsky and Nielsen (12) and later Miller and Baumann (13) have shown that the amount of xanthurenic acid excreted in the urine by rats kept on a diet rich in tryptophane depended on the vitamin B₆ present in the diet. More xanthurenic acid is excreted when the diet is deficient in vitamin B₆. Huff and Perlzweig (14) and later Johnson *et al.* (15) have shown that 4-pyridoxic acid is the main metabolic product of vitamin B₆ appearing in human urine. In our experiments the rats were placed in metabolism cages fitted

TABLE II
Urinary Excretion Studies on Vitamin B₆-Deficient Diets

	4-Pyridoxic acid excreted during 24 hrs.	Xanthurenic acid excretion per gm. food ingested
	γ	γ
Sucrose-basal + 18% fibrin.....	36	815
Dextrin-basal + 18% "	54	267

with glass funnels, and urine was collected for a period of 24 hours. Xanthurenic acid was estimated in a suitable aliquot of the urine sample, essentially according to the method of Miller and Baumann (13). After the conversion to lactone, 4-pyridoxic acid was estimated fluorometrically in the Coleman photofluorometer (14). The results, given in Table II, show that more 4-pyridoxic acid and less xanthurenic acid are excreted by animals on the dextrin diet than by those on the sucrose diet, indicating that the former animals are obtaining more vitamin B₆ than the latter. Since the two rations are equally deficient in vitamin B₆, the supposition is that the intestinal flora are stimulated to produce the extra vitamin B₆ in animals fed the dextrin diet.

From these results it is evident that dextrin cannot be used as a source of carbohydrate in the deficient ration and that blood fibrin is better than casein as the protein component of the ration. Experiments to determine the influence of biotin, inositol, and folic acid on the growth of rats receiving this deficient ration were carried out next. The results are given in Table III. Folic acid did not have any significant effect when added at a level of 25 γ per 100 gm. of ration. However, on rations with casein as the pro-

tein, the addition of biotin and inositol appeared to have a slight stimulating effect. These substances were consequently added to all the subsequent deficient rations.

The diet finally selected for the bioassay of vitamin B₆ has the following composition: sucrose 75 gm., blood fibrin 18 gm., Salts 4, 4 gm., corn oil 3 gm., thiamine 0.2 mg., riboflavin 0.3 mg., nicotinic acid 2.5 mg., calcium pantothenate 2 mg., 2-methyl-1,4-naphthoquinone 1 mg., inositol 10 mg., choline chloride 100 mg., and biotin 0.01 mg., per 100 gm. of diet. Halibut liver oil diluted 1:2 with corn oil was fed at a level of 2 drops per week, with α -tocopherol included at 0.5 mg. per drop. After a depletion period

TABLE III
Influence of Biotin, Inositol, and Synthetic Folic Acid on Growth of Vitamin B₆-Deficient Rats

Diet No.		Growth gain per wk. and range; average of 4 wks. growth
		gm.
1	Sucrose-basal + 18% casein without inositol and biotin	7 (6- 8)
2	Same as (1) + inositol, 10 mg. per 100 gm.	8 (7- 8)
3	" " (1) + biotin, 20 γ per 100 gm.	8 (7-10)
4	" " (1) + inositol and biotin	9 (8-10)
5	Sucrose-basal + 18% fibrin + biotin + inositol	5 (4- 5)
6	Same as (5) + <i>p</i> -aminobenzoic acid + synthetic folic acid, 25 γ per 100 gm. ration	5 (4- 7)
7	Sucrose-basal + 18% fibrin + 50 γ per 100 gm. pyridoxine HCl with inositol and biotin	20 (18-21)
8	Same as (1) + synthetic folic acid, 25 γ per 100 gm., + <i>p</i> -aminobenzoic acid + 50 γ per 100 gm. pyridoxine HCl	21 (18-24)

of 2 weeks, the rats are weighed, divided evenly with respect to weights into groups of three, and placed on diets containing different amounts of vitamin B₆. Fig. 1 shows the average weight gain per week plotted against increasing concentration of pyridoxine hydrochloride in the ration. The growth response is approximately linear up to 75 γ of pyridoxine hydrochloride for 100 gm. of diet. Twice this amount was required to permit maximum growth of over 30 gm. per week.

Comparison of Activities of Pyridoxine, Pyridoxamine, and Pyridoxal—Snell and Rannefeld (16) compared the activities of the members of the vitamin B₆ group by feeding them orally, separate from the ration, to deficient rats. They found that pyridoxine, pyridoxamine, and pyridoxal possessed equal activities within the limits of experimental error. Miller and Baumann (17) later reported that pyridoxamine and pyridoxal were

much less active than pyridoxine for mice and rats. The vitamin supplements in their experiments were added to the ration, a procedure which they found did not result in any destruction of pyridoxamine or pyridoxal. Luckey *et al.* (18) in experiments with chicks also found pyridoxamine and pyridoxal less active than pyridoxine. In view of these conflicting results the following experiments were carried out to test the potencies of the three compounds. Pyridoxine hydrochloride, pyridoxamine dihydrochloride, and pyridoxal hydrochloride were administered by three different methods. In one group the three compounds were each mixed in 100 gm. of ration

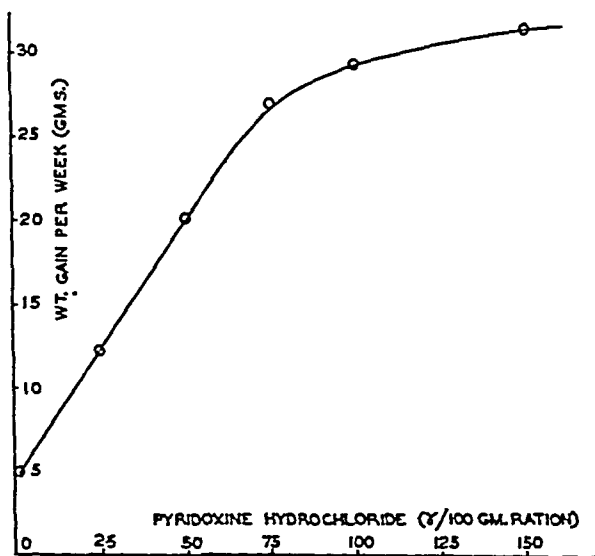


FIG. 1. Growth response of rats to pyridoxine hydrochloride on the improved ration.

at equimolar levels equivalent to 50 γ of pyridoxine hydrochloride. In the second group, the rats were fed daily molar supplements equivalent to 5 γ of pyridoxine hydrochloride by dropper. In the third group, these same amounts were injected intraperitoneally. The results (Table IV) show a marked decrease in the growth of rats on pyridoxamine and pyridoxal when fed in the ration, whereas all compounds were equally active when fed by dropper or by intraperitoneal injection. Similar results (Table IV) were obtained with chicks. Day-old white Leghorn chicks were fed the deficient ration (Table IV, foot-note) and were kept in heated cages with raised screen bottoms, seven chicks being used in each group.

All the chicks on the deficient diet died in 2 weeks without gaining any appreciable weight. The results obtained when pyridoxine, pyridoxamine,

TABLE IV
Comparison of Activities of Pyridoxine Hydrochloride, Pyridoxamine Dihydrochloride, and Pyridoxal Hydrochloride

	Average weekly gain of rats in gm.			Average weight of chicks* at 3 wks. in gm.		
	In ration, 50 γ per 100 gm.	Fed by dropper, 5 γ per rat per day	Intra-peritoneal injection, 5 γ per rat per day	In ration, 100 γ per 100 gm.	Fed by dropper, 7.5 γ per chick per day	Intra-peritoneal injection, 7.5 γ per chick per day
Pyridoxine HCl... ..	20	24	22	124	106	111
Pyridoxamine 2HCl (equimolar)... ..	15	21	21	74	103	107
Pyridoxal HCl . . .	14	25	26	91	111	112

* The basal ration used for the production of vitamin B₆ deficiency in chicks had the following composition: cerelose 61 gm., blood fibrin 18 gm., gelatin 10 gm., Salts 4, 5 gm., CaHPO₄·2H₂O 1 gm., L-cystine 0.3 gm., thiamine hydrochloride 0.3 mg., riboflavin 0.6 mg., calcium pantothenate 2.0 mg., choline chloride 150 mg., nicotinic acid 5.0 mg., biotin 0.02 mg., inositol 100.0 mg., synthetic folic acid 0.125 mg., vitamin D₂ 0.004 mg., α-tocopherol 0.03 mg., 2-methyl-1,4-naphthoquinone 0.05 mg., vitamin A 1700 i.u. This ration differed from the one used by Luckey *et al.* (18) in that cerelose replaced dextrin and synthetic folic acid was substituted for norit eluate of 1:20 liver powder.

TABLE V
Comparative Activities for Rats of Pyridoxine Hydrochloride and Synthetic Codecarboxylase

	In ration		Intraperitoneal injection	
	Amount fed* γ per 100 gm.	Average gain and range gm. per wk.	Amount injected* γ per rat per day	Average gain and range gm per wk.
Pyridoxine HCl	40	16 (12-20)	2.5	15 (14-16)
Synthetic codecarboxylase (pyridoxal phosphate)	125	11 (10-12)	7.8	15 (14-18)

* The preparation of pyridoxal phosphate used contained 32 per cent of pyridoxal, and was consequently used at levels equimolar with pyridoxine. The barium present was removed as barium sulfate before administration.

and pyridoxal were mixed in 100 gm. of ration at a level of 100 γ, given by dropper at 7.5 γ per chick per day and injected intraperitoneally, at the same level are given in Table IV. There was a marked decrease in the

growth of chicks in the first group when pyridoxal and pyridoxamine were mixed in the ration, while the growth was approximately the same in all cases in which vitamin supplements were fed by dropper or injected intraperitoneally. The lower activity of pyridoxal and pyridoxamine when fed in the ration cannot be attributed to destruction during storage, since yeast assays on the amounts of vitamins present in the rations under the storage conditions used (not more than 10 days at 4°) showed no destruction of pyridoxine, pyridoxal, or pyridoxamine.

Codecarboxylase (phosphorylated pyridoxal), which has been synthesized and purified as the barium salt by Gunsalus and coworkers (19), was compared with pyridoxine hydrochloride for its relative activity for the rat. The coenzyme,¹ which contained 32 per cent of pyridoxal after hydrolysis as determined by spectrum analysis (19), was mixed with the ration or injected intraperitoneally in quantities calculated to furnish amounts of pyridoxal equimolar with the pyridoxine supplied to the control groups. The results are given in Table V. Like pyridoxal hydrochloride, codecarboxylase was less active than pyridoxine when mixed in the ration, but had the same activity as pyridoxine when injected intraperitoneally.

DISCUSSION

Snell (5) showed by differential microbiological assay methods with yeast (*Saccharomyces carlsbergensis*), *Streptococcus faecalis*, and *Lactobacillus casei* that natural materials contain varying proportions of pyridoxine, pyridoxamine, and pyridoxal. These compounds possess equivalent activities towards yeast and also towards animals, if fed by a dropper or by injection. However, if the supplements are mixed in the ration, pyridoxamine and pyridoxal show considerably less activity than pyridoxine for both rats and chicks. The explanation for these results is at present unknown. A possible explanation is that pyridoxal and pyridoxamine are more susceptible to destruction or utilization by intestinal bacteria than is pyridoxine. When exposed to bacterial action, as when fed in the ration, less of these compounds might be available for the animal. When fed by a dropper, absorption is rapid, and such influences could not enter to alter the true comparative activities of the three compounds. In support of this hypothesis are the known facts that lactic acid bacteria constitute a large proportion of the total intestinal flora (20, 21), that several of these organisms require vitamin B₆ for growth (16), and that, although these organisms utilize pyridoxal and pyridoxamine readily, they are in general unable to utilize pyridoxine (16). Whatever the true explanation, it is evident that when natural materials in which most of the vitamin B₆ is

¹ We are indebted to Dr. I. C. Gunsalus for a pure specimen of the barium salt of pyridoxal phosphate.

present as pyridoxal and pyridoxamine are fed with the ration, a considerably lower figure for the vitamin B₆ content might result from rat assay than would be obtained by yeast assay. When pyridoxine is the major constituent of the B₆ group present, the same values should be obtained. Considering further that methods of extraction of vitamin B₆ from natural materials for microbiological and chemical assay are not entirely satisfactory, it is evident that bioassay with the rat is still of great value in assessing the vitamin B₆ activity of dietary constituents used in animal nutrition.

SUMMARY

A deficient diet for the assay of vitamin B₆ with the rat is described which is based on sucrose as the carbohydrate and blood fibrin as the protein. In the absence of vitamin B₆, the diet gives the least growth in weanling rats of any tried; maximum growth (over 30 gm. per week) is obtained when 150 γ of pyridoxine hydrochloride are added to 100 gm. of ration. When dextrin is substituted for sucrose, considerable growth occurs on the deficient diet. On the dextrin diet, urinary excretion of xanthurenic acid is decreased, while the amount of 4-pyridoxic acid excreted increases. The addition of sulfathalidine to such a ration eliminated its growth-promoting effects; growth is resumed if vitamin B₆ is fed. These results indicate that on the dextrin diet intestinal flora synthesize considerable amounts of vitamin B₆, which is then utilized by the rat.

A standard curve which is approximately linear up to 75 γ of pyridoxine hydrochloride in 100 gm. of ration has been obtained on the improved basal diet. The relative activities of pyridoxine, pyridoxamine, and pyridoxal vary with the way in which the supplements are given. When mixed in the ration, pyridoxamine and pyridoxal are less active than pyridoxine; when given by dropper or injected intraperitoneally, they all possess equivalent activities. Similar results have been obtained with chicks.

The growth-promoting potency of synthetic pyridoxal phosphate (code-carboxylase) corresponds to its pyridoxal content. Like pyridoxal, it is less active than pyridoxine when mixed in the ration, but is equally active when injected intraperitoneally.

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CHEMICAL DETERMINATION OF RIBOFLAVIN*

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The increased interest in the riboflavin content of both foods and feeds makes it highly desirable that we have a rapid and accurate method for determination of this vitamin which is generally applicable. While the microbiological procedure of Snell and Strong (1) has been found to be very accurate, it has the disadvantage of requiring several days to obtain results. Furthermore, many laboratories are not equipped to conduct microbiological determinations. There exists, therefore, a need for an accurate chemical method which can be used to analyze a large number and variety of samples in a short period of time.

The chemical method of Hodson and Norris (2) has been found to give satisfactory results on certain materials, but, when substances containing highly fluorescent non-riboflavin pigments are analyzed, erroneously high results are obtained by this procedure. The method of Hodson and Norris has been modified several times during the past 5 years. The major modification consists of the substitution of a procedure of oxidation of interfering pigments by the use of potassium permanganate for the reduction procedure used previously. The potassium permanganate oxidation of interfering pigments was probably first used by Koscharka (3) in his early studies of the lyochromes. Its use in an assay procedure was first reported by Ferrebee (4) in his method for the determination of riboflavin in urine. This, together with certain modifications in technique, has resulted in the development of an accurate chemical assay method for riboflavin. The method, as described in this report, has been used by chemists in several different laboratories on a wide variety of materials with very satisfactory results. In the extensive studies of the riboflavin content of milk and milk products and of dried leguminous seeds conducted by Daniel and Norris (5, 6) this

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method was found to give values which were in excellent agreement with those obtained microbiologically.

Method

Reagents—

Acid-acetone solution. 250 ml. of N HCl are diluted to 1 liter with acetone.

Trisodium phosphate solution. 65 gm. of $Na_3PO_4 \cdot 12H_2O$ are dissolved and made to 1 liter with distilled water.

Sodium hydrosulfite solution. 5 gm. of $Na_2S_2O_4$ are dissolved in 100 ml. of 0.2 M disodium phosphate solution, 1 to 2 gm. of Celite analytical filter aid¹ are added, and the solution is filtered through No. 44 Whatman filter paper. The solution is placed in an ice bath. The sodium hydrosulfite is unstable and should not be used after about 4 hours.

Glacial acetic acid.

Potassium permanganate solution. A 4 per cent solution of potassium permanganate is freshly prepared each week.

Hydrogen peroxide solution. A 3 per cent solution is prepared by diluting a 30 per cent solution of hydrogen peroxide 1:10 with distilled water.

Standard riboflavin solution. Riboflavin of the highest purity obtainable is dried to constant weight *in vacuo* at 60°. From this a stock solution is prepared by dissolving 50 mg. in distilled water and making up to a volume of 1 liter. The solution should be preserved in a cool place and protected from the light. The addition of a few drops of glacial acetic acid will insure an acid pH and help stabilize the solution. This standard riboflavin solution is called Solution I.

*Extraction Procedure—*A 1 to 5 gm. finely ground sample of the unknown is weighed into a 250 ml. red Erlenmeyer flask² and 100 ml. of the acid-acetone solution are added. After thorough mixing, the mixture is gently refluxed for 1 hour. The extract is cooled to room temperature, after which it is brought to pH 6.6 to 6.8 with trisodium phosphate solution. Sufficient water is added to make the total volume of liquid 200 ml. After standing 5 minutes the mixture is filtered through No. 12 Whatman fluted filter paper. The first 10 to 20 ml. of filtrate are discarded. A 10 to 50 ml. aliquot of the filtrate is pipetted into a 200 ml. volumetric flask. The pH of this solution is adjusted to 3.7 with glacial acetic acid. 2 ml. of 4 per cent potassium permanganate per 10 ml. of filtrate are added, mixed, and allowed to stand for 3 minutes. At the end of 3 minutes the solution of 3 per cent hydrogen peroxide is added until the color of the permanganate is

¹ Obtained from Johns-Manville.

² Pyrex Brand Lifetime Red glassware obtained from the Corning Glass Works. Corning, New York.

changed from purple to light brown. The solution is made to volume, 1 to 2 gm. of Celite analytical filter aid are added, and the solution is filtered through No. 44 Whatman filter paper. The first 10 to 20 ml. of filtrate are discarded in order to eliminate turbidity in the filtrate caused by a few small particles which sometimes pass through the filter paper before the filter aid has formed an efficient mat. A known volume of this clear solution is then pipetted into the optical cell for the fluorometric determination.

20 ml. of Solution I are dissolved in 100 ml. of acid-acetone solution neutralized to pH 6.6 to 6.8 with trisodium phosphate solution and made to a volume of 200 ml. with water. 40 ml. of this solution are taken to pH 3.7 with glacial acetic acid, treated with 8 ml. of potassium permanganate solution for 3 minutes, almost decolorized with 3 per cent hydrogen peroxide, made to a volume of 200 ml., and filtered through No. 44 Whatman filter paper after addition of Celite filter aid. This solution is used as the standard in determining the riboflavin content of the unknowns. This standard riboflavin solution is called Solution II. The pH of this solution should be identical with that of the unknowns. If carried out as above it will be approximately pH 4.0. This solution may be kept in a red flask in the refrigerator for at least a week.

Fluorometric Determination—A distilled water blank is placed in the fluorophotometer and the galvanometer is set at zero. 1 ml. of the Solution II (1 γ per ml.) is added to 15 ml. of distilled water and this reading is noted. This is called reading *S*. Then 15 ml. of the unknown solution are pipetted into the glass cell and a reading (*A*) is made. The glass cell is removed from the path of light and 1 ml. of the Solution II is added and a second reading (*B*) made. After this the riboflavin in the solution is reduced to the non-fluorescing leuco form by adding 1 ml. of the sodium hydrosulfite solution and a third reading (*C*) is made. Reading *S* is taken again to make sure that the light intensity has not changed during the above operations.

Calculation of Results—The micrograms of riboflavin per ml. of unknown solution are calculated as follows:

$$(1) \quad \frac{A - C}{B - A} D = \text{micrograms riboflavin per ml. unknown solution (uncorrected)}$$

where *A* = fluorescence reading of 15 ml. aliquot of unknown solution; *B* = fluorescence reading of unknown solution after addition of 1 ml. of standard riboflavin Solution II (total volume 16 ml.); *C* = fluorescence reading after further addition of 1 ml. of sodium hydrosulfite solution (total volume 17 ml.); *D* = micrograms of added riboflavin per ml. of solution at the time reading *B* is taken ($D = 1 \gamma/16 \text{ ml.} = 0.0625 \gamma \text{ per ml.}$).

However, since reading *A* was taken in 15 ml. while reading *B* was taken after dilution of the solution to 16 ml., and reading *C* was taken when the

total volume of the solution was 17 ml., readings B and C must be corrected to the values that they would have been if the readings had been made in 15 ml. of solution in each case. This is accomplished by the use of correction factors y and z ($y = 16/15 = 1.067$ and $z = 17/15 = 1.133$) as shown in Equation 2.

$$(2) \quad \frac{A - Cz}{By - A} D = \text{micrograms riboflavin per ml. unknown solution (corrected)}$$

Since Equation 2 gives the micrograms of riboflavin per ml. of unknown solution, the amount of riboflavin per gm. of sample is obtained by multiplying the result of Equation 2 by the dilution factor f ($f = 400$ if the original extract of a 5 gm. sample is diluted to 200 ml. and a 20 ml. aliquot of this is further diluted to 200 ml.). Thus the final equation, which is used in determining the micrograms of riboflavin per gm. of sample, is

$$(3) \quad \frac{A - Cz}{By - A} Df = \text{micrograms riboflavin per gm. unknown}$$

DISCUSSION

It has been found that a solution containing both acid and acetone is most effective in the extraction of riboflavin. The acetone acts to flocculate colloidal suspensions and, since it is an efficient eluting agent for riboflavin, it also prevents adsorption of the vitamin from solution by the proteins.

Experience has shown that in the case of highly pigmented materials, such as dried distillers' grains and fermentation products, the amount of potassium permanganate used may be doubled without causing destruction of riboflavin.

It has been found that wheat products tend to cause the solution to froth when hydrogen peroxide is added to the permanganate solution. This frothing can be dispelled by addition of a few drops of acetone.

In an early study conducted at this laboratory it was found that the fluorescence of riboflavin varies with the pH of the solution according to the curve presented in Fig. 1. Upon examination of this curve it is apparent that at pH values near neutrality a slight change in the pH of the riboflavin solution could cause an appreciable change in the intensity of fluorescence of the vitamin, while at pH 4.0 solutions of riboflavin may be compared fluorometrically without the necessity of accurately establishing the pH of each solution. In view of this advantage it was found advisable to carry out the fluorescence measurements at pH 4.0.

The use of this pH made it necessary to use 0.2 M disodium phosphate to buffer the sodium hydrosulfite solution used in the final reduction of the riboflavin, since at this pH the sodium bicarbonate buffer used by Hodson

and Norris decomposed, forming bubbles of carbon dioxide which interfered with the final fluorescence reading.

The use of Celite analytical filter aid has been found necessary in order to obtain solutions absolutely free of cloudiness. Cloudiness in the final solutions used for making the fluorometric readings may cause a significant error in results.

The effect of unstable interfering pigments upon the fluorescence of the riboflavin in an aliquot of the unknown solution is eliminated by the oxidation with potassium permanganate. However, certain materials contain stable interfering pigments which absorb, to some extent, the activating and fluorescent light and thus interfere with an accurate measurement of the

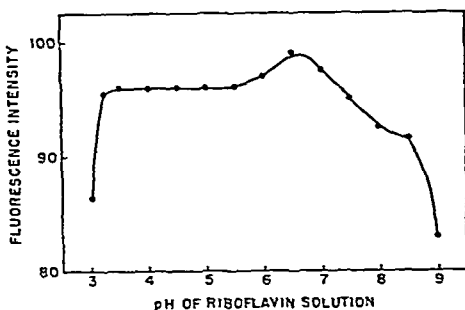


FIG. 1. Influence of pH upon the fluorescence of a standard solution of riboflavin containing 0.1 γ of riboflavin per ml. of solution.

riboflavin fluorescence. This difficulty is overcome by adding a known amount of a standard solution of riboflavin to the unknown solution. The stable interfering pigments then act equally upon the fluorescence of the standard riboflavin solution and the fluorescence of the riboflavin in the unknown solution. The riboflavin in the unknown solution can then be determined accurately by comparing its fluorescence with that of the standard riboflavin under the same conditions. The effect of stable substances which also show green fluorescence is eliminated by the use of the sodium hydrosulfite. Sodium hydrosulfite reduces the riboflavin to a non-fluorescing form without changing the fluorescence of the interfering substances. This residual fluorescence is subtracted from the total fluorescence of the unknown solution in order to determine the fluorescence due to riboflavin.

Comparison of Chemical Method with Microbiological Method

The riboflavin content of a variety of foods and feedstuffs is presented in Table I. The riboflavin values presented are analyses of single samples.

TABLE I
Riboflavin Content of Foods and Feeds

Material	Fluorometric method	Microbiological method
	γ per gm.	γ per gm.
Whole yellow corn.....	1.00	1.05
Table corn-meal.....	0.80	
Fancy ".....	1.05	1.00
Feed ".....	1.40	1.40
Corn gluten meal.....	2.30	2.50
" " feed.....	4.60	4.60
Dried distillers' grains, Sample 1.....	2.70	2.70
" " " " 2.....	7.50	7.50
Whole wheat.....	1.85	1.65
Wheat bran.....	3.00	3.20
" germ.....	4.25	
Standard wheat middlings.....	3.55	3.80
Wheat flour middlings.....	2.40	2.50
Whole wheat flour.....	1.40	1.40
Patent " ".....	0.50	0.50
Enriched wheat flour.....	3.00	3.10
" bread.....	3.50	3.40
Whole oats.....	1.45	1.20
Rolled ".....	0.75	
Pulverized oats.....	1.70	1.70
Whole barley.....	1.50	
Brewers' dried grains.....	1.30	
Soy bean oil meal (solvent).....	3.00	3.25
" " " " (expeller).....	3.25	
Linseed oil meal.....	2.40	2.40
Cottonseed ".....	3.55	
Copra meal.....	5.20	5.70
Peanut ".....	4.90	4.80
Fermentation product, Sample 1.....	101.00	100.00
" " " " 2.....	258.00	265.00
" " " " 3.....	38.90	39.30
Whole milk.....	1.69	1.71
Dried skim milk.....	16.60	16.00
" whey.....	22.20	22.20
" buttermilk.....	29.20	28.30
Dehydrated alfalfa meal.....	15.90	15.70
Dried cereal grass.....	15.65	15.60
Tomato pomace.....	3.65	
Meat scrap.....	4.60	4.40
Fish meal.....	7.30	7.60
Liver ".....	57.80	61.20
Dried brewers' yeast, Sample 1.....	36.20	38.70
" " " " 2.....	17.80	18.50
Commercial feed mixture.....	4.30	4.20

When an appreciable variability in riboflavin content of different samples of any given food or feedstuff was found, the value reported is that of a sample having approximately the average riboflavin content of that particular material. However, certain materials have been found to vary so widely in riboflavin content that the amount of the vitamin present in any one sample cannot be predicted with any degree of accuracy. This is especially true of materials such as meat scrap, fish meal, liver meal, dried brewers' yeast, dried distillers' grains, and alfalfa meal, in which the composition of each sample may vary considerably. The method of processing may also contribute to variations in riboflavin content of these materials.

The results presented in Table I demonstrate that the chemical method for determination of riboflavin as described in this report gives values for a wide variety of food materials which are in excellent agreement with those obtained microbiologically. Since this is true in the case of highly pigmented materials such as fermentation products, alfalfa meal, and liver meal as well as in the case of the less highly pigmented materials, it is evident that the chemical method is generally applicable.

SUMMARY

The chemical method of Hodson and Norris for the determination of riboflavin has been modified so as to make it generally applicable.

A number of different food materials have been assayed for riboflavin by the method as described in this report and by the microbiological method of Snell and Strong. A comparison of the values obtained by these two procedures shows that the chemical method gives results which are in excellent agreement with those obtained microbiologically.

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THE UTILIZATION OF A COLOR CORRECTION EQUATION WITH THE KOBER REAGENT FOR THE ESTIMATION OF THE ESTROGENS IN HUMAN URINE WITH LOW ESTROGEN CONTENT

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We have shown (1) that satisfactory Kober tests (i.e., an $L-520\text{ m}\mu$ to $L-420\text{ m}\mu$ ratio greater than 3) can be obtained for the estrone and estriol fractions from the 10th week and for estradiol fractions from the 24th week following the last catamenia by our method for the fractionation and photometric estimation of the estrogens in human pregnancy urine. In fractions of lower estrogenic titer, a brown color is produced with the Kober reagent by the non-estrogenic chromogens which contaminate the estrogen factors. Some form of correction to reduce the error of overestimation caused by this brown color is necessary.

Bachman and Pettit (2) considered techniques proposed to correct for the overestimation caused by the Kober reagent in crude urine residues (3-5) to be inadequate but presented no evidence to support this view. Fraser *et al.* (6) suggested the use of a color correction equation with the Zimmermann test (7) for neutral 17-ketosteroids in crude urine extracts, and Talbot *et al.* (8) discussed the rationale for the use of such an equation and established its validity.

To correct for the overestimation of estrogens in our chromatographic filtrate residues by the Kober reagent we have used a somewhat similar equation of the form

$$C_x = \frac{L-520\text{ m}\mu \text{ mixture} - B_x(L-420\text{ m}\mu \text{ mixture})}{K_x(1 - A_x B_x)}$$

in which the symbols have the following meaning: C_x = the weight of the estrogen component of the test in micrograms (C_o = estrone, C_d , estradiol, C_i , estriol); L = 2 - the log of the galvanometer reading; A_x = the ratio of $L-420\text{ m}\mu$ to $L-520\text{ m}\mu$ for the Kober color test on pure estrogen; B_x = the ratio of $L-520\text{ m}\mu$ to $L-420\text{ m}\mu$ for the interfering chromogens; and K_x = the calibration constant for the Kober test on pure estrogen. Because the identities of interfering chromogens of urine extracts appearing in our chromatographic fractions have not yet been established, it becomes necessary to derive sufficient statistical data from essentially estrogen-free urine extracts to evaluate the B_x values for our equation.

The present paper is concerned with a study of the variations in the spectrophotometric characteristics (at 420 and 520 $m\mu$) of the brown color produced by the Kober reagent with our chromatographic filtrate residues from essentially estrogen-free urine extracts and the utilization of these data for estimating by means of a color correction equation the estrogen content of human urine of low estrogen titer.

EXPERIMENTAL

Reagents and apparatus were those described in a previous publication (1).

Procedure

Preparation of Urine Residue Suitable for Liquid Chromatogram—The residue was prepared essentially as described (1) with the following modifications. The residue from the butyl alcohol extract was dissolved in 100 ml. of 0.5 N sodium hydroxide and diluted with distilled water to a 200 ml. volume. 30 ml. of concentrated hydrochloric acid were then added and the aqueous extract was boiled under a reflux for 15 minutes. After cooling under running tap water, the extract was shaken three times with 100 ml. amounts of ethyl ether. The combined ether extracts were washed twice with 25 ml. of 9 per cent sodium bicarbonate solution and once with 25 ml. of distilled water. The ether solution, which should be free from any insoluble material at this point, was then extracted three times with 100 ml. and once with 50 ml. volumes of normal sodium hydroxide. The combined sodium hydroxide extract was acidified (to litmus paper) with hydrochloric acid and reextracted three times with 100 ml. volumes of ether. The combined ether extracts were washed twice with 40 ml. volumes of 9 per cent sodium bicarbonate and once with 40 ml. of distilled water. The ether residue was stored as described in our previous publication (1).

Preparation of Liquid Chromatogram—The method previously described (1) was followed with one exception: the 50 ml. benzene developer was omitted and the filtrate which was collected up to the time of addition of the 2 per cent methanol-benzene was discarded.

Results

Summary of Spectrophotometric Data on Chromatographic Filtrate Residues Treated with Kober Reagent—In deriving the Kober calibration constants for crystalline estrone ($K_o = 0.00837$), estradiol ($K_d = 0.00523$), and estriol ($K_i = 0.00737$) previously reported by us (1), a study (unpublished) was also made of the ratio of $L-520\ m\mu$ to $L-420\ m\mu$ for each of these tests. The average ratio of $L-520\ m\mu$ to $L-420\ m\mu$ for the estrone series was $10 \pm$

1.6, for α -estradiol 7.5 ± 1.0 , for estriol 10.2 ± 0.9 . The reciprocals of these average values constitute the A_z values in Table I. Inspection of the general equation reveals that, since B_z varies between 0.54 and 0.77, any expected variation within the above limits from the average A_z values may be ignored in evaluating C_z , the weight of estrogen component in micrograms.

The same situation with respect to the variation in our B_z values (the ratio of L -520 $m\mu$ to L -420 $m\mu$ for the average brown color) does not

TABLE I

Spectrophotometric Data on Pure Estrogens and on Residues from Series of Thirty Essentially Estrogen-Free Urine Extracts, Treated with Kober Reagent

Description of measurement	Chromatographic fraction tested		
	Estrone	Estradiol	Estriol
A_z , i.e. $\frac{L-420 \text{ } m\mu}{L-520 \text{ } m\mu}$ for pure Kober color	0.10	0.13	0.10
K_z , i.e. calibration constant for pure Kober product	0.00837	0.00523	0.00737
L -520 $m\mu$ for non-estrogenic chromogens	0.478 (0.137-0.852)	0.324 (0.092-0.573)	0.293 (0.077-0.602)
L -420 $m\mu$ for non-estrogenic chromogens	0.815 (0.250-1.540)	0.520 (0.143-0.894)	0.425 (0.116-0.876)
B_z , i.e. $\frac{L-520 \text{ } m\mu}{L-420 \text{ } m\mu}$ for non-estrogenic chromogens	0.59 (0.54-0.64)	0.63 (0.57-0.70)	0.69 (0.61-0.77)
$K_z(1 - A_z B_z)$	0.0079	0.0048	0.0069
Uncorrected estrogen content, γ^*	57 (16 - 100)	62 (18 - 110)	39 (10 - 81)
Corrected estrogen content, γ^\dagger	0.33 ± 7.0	0.80 ± 9.0	0.40 ± 5.0

* Calculated by dividing L -520 $m\mu$ by K_z .

† Calculated by use of the color correction equation.

necessarily hold true, since B_z appears both in the numerator and denominator of our equation. Spectrophotometric data from a series of thirty 24 hour essentially estrogen-free urine specimens from preadolescent (8 to 10 years old) girls and bilaterally ovariectomized adult females were compiled in order to derive an average B_z value for each chromatographic fraction and to test the significance of its anticipated variation from the average value. These data are summarized in Table I.

The chromatographic filtrate residues prepared from these specimens were tested with the Kober reagent as previously described (7). Each Kober color product was uniformly washed with ethyl acetate but the

photometric density measurements (L values) here recorded were made before such treatment. In most instances half the total filtrate residue was used for a single color test. However, the L -520 and L -420 $m\mu$ values summarized in Table I represent the complete 24 hour total for each fraction. Analysis of the data in Table I reveals that, whereas the photometric density values of the brown Kober color for all three chromatographic fractions covered a wide range in the series investigated, the B_z value (ratio of L -520 $m\mu$ to L -420 $m\mu$) for each fraction never exceeded 12 per cent deviation from the average ($B_o = 0.59 \pm 0.05$, $B_d = 0.63 \pm 0.07$, $B_i = 0.69 \pm 0.08$). A limited number of two successive chromatograms on the same urine extract has yielded results in agreement with these B_z values. Since the magnitude of this deviation from an average B_z value appears to be the limiting factor in the accuracy obtainable by use of the equation, we have calculated with the aid of our equation the amount of estrogen theoretically present in each of the thirty essentially estrogen-free urine specimens. This corrected estrogen content of our chromatographic fractions for each of the thirty urine specimens did not exceed $\pm 7 \gamma$ of estrone, $\pm 10 \gamma$ of estradiol, and $\pm 5 \gamma$ of estriol. It was assumed (9-11) that each of the 24 hour urine specimens actually contained less than 1 γ of estrogen in each chromatographic fraction. Therefore these figures represent the maximum error inherent in any 24 hour urine determination, due to the extent to which successive extracts differ from the calculated average B_z values.

The above data can be utilized for analyzing mixed Kober colors only if the true Kober color and the brown contaminating color develop independently in the presence of each other. Table II contains data from a number of Kober tests designed to show this independent development of color. Aliquots from the same chromatographic filtrate residue from a 24 hour essentially estrogen-free urine specimen, with and without the addition of known amounts of the appropriate estrogen, were treated with the Kober reagent. The results demonstrate that non-estrogenic impurities present in our chromatographic filtrate residues do not interfere significantly with the Kober reaction with pure estrogens and *vice versa*.

Recovery of Estrogens Added to Essentially Estrogen-Free Urine Extracts Immediately after Hydrolysis—Table III summarizes the data from a number of recovery experiments which were carried out to test the quantitative features of our fractionation and photometric estimation technique when applied to successive 24 hour specimens of castrate female urine enriched with small amounts of crystalline estrogens, immediately after hydrolysis. Since the estrogen was added to the equivalent of a 24 hour urine specimen, each estrogen estimation is subject to the limitations imposed by our average B_z values; i.e., estrone $\pm 7 \gamma$, estradiol $\pm 10 \gamma$, and estriol $\pm 5 \gamma$.

Estrone and α -estradiol are recovered in the appropriate filtrate fractions in the range 80 to 100 per cent; estriol is recovered in the range of 60 to 70 per cent, which is somewhat lower than the 75 to 83 per cent recovery reported (1) for urines with higher estriol titer. This loss of estriol in the purification procedure is greater than is desirable, but it is reasonably constant and therefore does not seriously interfere with the significance of the estimation.

TABLE II

Comparison of Determined and Theoretical Values for L-520 m μ and L-420 m μ for Kober Color Products from Our Chromatographic Filtrate Fractions, before and after Addition of Known Amounts of Estrogen to Essentially Estrogen-Free Urinary Residues

Each test was performed on the equivalent of half of a 24 hour urine from an ovariectomized human female.

Experiment No.	Chromatographic filtrate fraction*	Estrogen added	L-520 m μ		L-420 m μ	
			Determined	Theoretical	Determined	Theoretical
		γ				
1	1	None	0.204		0.377	
	1	30 O.	0.482	0.455	0.420	0.402
2	1	None	0.119		0.204	
	1	60 O.	0.648	0.621	0.228	0.254
3	2	None	0.319		0.534	
	2	30 D.	0.491	0.476	0.549	0.554
4	2	None	0.204		0.347	
	2	60 D.	0.512	0.518	0.372	0.388
5	3	None	0.125		0.176	
	3	25 T.	0.310	0.309	0.191	0.194
6	3	None	0.211		0.250	
	3	50 T.	0.561	0.579	0.268	0.287

O. = estrone; D. = α -estradiol; T. = estriol.

* Fraction 1 = 2 per cent methanol-benzene; Fraction 2 = 5 per cent methanol-benzene; Fraction 3 = 30 per cent methanol-benzene.

Estimation of Estrogens in Urine Specimens with Low Estrogen Titers—The results of some of our preliminary studies which were made to determine the potentialities and limitations of our colorimetric estimations of the estrogens in urine with low estrogen titer are given in Table IV. Experiment 1 gives the results of estrogen determinations throughout a normal female menstrual cycle. Complete 48 hour urine specimens were collected during the entire cycle with the exception of the catamenia. The 48 hour specimen collected on the 19th and 20th days was used for a pregnanediol glucuronide determination according to the method of Venning (12). 6 mg. were obtained, which constitutes a positive reaction. It would

TABLE III
*Estrogen Titrers for Equivalent of 24 Hour Essentially Estrogen-Free Urine Specimens
 Enriched with Known Amounts of Crystalline Estrogens Immediately after
 Hydrolysis*

Experiment No.	Amount of estrogen added	Filtrate fraction No.*	Total estrogen found by Kober reagent
	γ		γ
1	120 O.	1	108
		2	13
		3	4
2	120 D.	1	10
		2	104
		3	8
3	100 T.	1	8
		2	12
		3	62
4	120 O.	1	106
	100 T.	2	19
		3	66
5	60 O.	1	50
	30 D.	2	36
	100 T.	3	70

O. = estrone; D. = α -estradiol; T. = estriol.

* Fraction 1 = 2 per cent methanol-benzene; Fraction 2 = 5 per cent methanol-benzene; Fraction 3 = 30 per cent methanol-benzene.

TABLE IV
Estrogen Content of Human Normal and Early Pregnancy Urine

Experiment No.		Estrogen content per 24 hrs.			
		Estrone	Estradiol	Estriol	Total
		γ	γ	γ	γ
1	Normal female cycle, 5-6 days	8	3	5	16
	" " " 7-8 "	6	4	7	17
	" " " 9-10 "	2	5	10	17
	" " " 11-12 "	23	20	18	61
	" " " 13-14 "	8	12	7	27
	" " " 15-16 "	6	6	18	30
	" " " 17-18 "	3	9	14	26
	" " " 21-22 "	12	8	3	23
	" " " 23-24 "	9	12	3	24
	" " " 27-28 "	3	1	-2	2
2	Pregnancy, 4-5 wks. after last catamenia	50	38	37	125
3	" " "	55	30	38	123
4	Pregnancy, 8-9 wks. after last catamenia	250	70	300	620
5	Normal male	2	4	1	7
6	" "	5	6	4	15
7	" "	5	4	2	11

appear from this preliminary study that the method is capable of detecting the midmenstrual elevation of estrogens but that it is of doubtful value for a considerable portion of the cycle because of the limitations imposed by the use of the average B_x values.

For female urine specimens collected as early as 1 week after the first missed catamenia in normal pregnancy, corrected estrone and estriol titers of sufficient magnitude were obtained to bring the error due to the use of a color correction equation within favorable limits (10 to 20 per cent). Experiments 2 and 3 in Table IV are representative of these findings. This evidence would suggest that the method might be a convenient substitute for the commonly used gonadotropic bioassay for pregnancy. Normal male urine specimens as indicated in Experiments 5 to 7 consistently yielded estrogen titers within the range associated with essentially estrogen-free specimens.

DISCUSSION

Duplicate Kober determinations on our chromatographic filtrate residues from essentially estrogen-free urine extracts have consistently been in satisfactory agreement as is obtainable with pure estrogens (6 per cent). Washing of these essentially estrogen-free Kober color products with ethyl acetate reduced by one-third to one-half the photometric density at 520 and 420 $m\mu$ without materially altering the B_x values (ratio of L -520 $m\mu$ to L -420 $m\mu$). This observation is further evidence that there is negligible estrogen present in the residue and is being investigated further as a possible means of evaluating the B_x value for each individual Kober test. It is recommended that the estrogen content of all Kober tests in which an L -520 $m\mu$ to L -420 $m\mu$ ratio of less than 6.0 is obtained be calculated by means of the color correction equation, since the error of overestimation approximates 10 per cent at this ratio and increases drastically with lower ratios.

SUMMARY

A color correction equation for the Kober reagent has been proposed and sufficient experimental data have been derived to evaluate the constants therein. By use of the color correction equation the overestimation of estrogens in a representative 24 hour urine specimen is reduced to $\pm 7 \gamma$ of estrone, $\pm 10 \gamma$ of estradiol, and $\pm 5 \gamma$ of estriol. The method is sufficiently sensitive to indicate the midmenstrual elevation of estrogen excretion in a normal female menstrual cycle.

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STUDIES ON THE INHIBITION OF ENZYME SYSTEMS INVOLVING CYTOCHROME *c**

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During the course of studies on the inhibition of the enzymatic oxidation of glutathione in the presence of added cytochrome *c*, it was noted that the type of inhibition observed (1) did not correspond with that previously reported for systems involving cytochrome *c*. An investigation was made to compare the action of a number of inhibitors on three systems in which cytochrome *c* is concerned; namely, the enzymatic oxidations of ascorbate, reduced glutathione, and succinate.

The exact nature of the enzyme catalyzing the reaction between cytochrome *c* and oxygen, by definition cytochrome oxidase, has not been fully elucidated. It was thought that a study of the action of a number of inhibitors on systems in which cytochrome oxidase was concerned might reveal whether it was an iron- or a copper-containing enzyme. In addition further information might be gained concerning the intermediate systems lying between the initial substrate and oxygen.

The results of this investigation indicate that the hydrogen donor-cytochrome *c*-cytochrome oxidase-oxygen system may be more complex than has been previously proposed, and involves both iron and copper. The action of several inhibitors is shown to differ markedly, depending on which hydrogen donor is used as the initial substrate.

EXPERIMENTAL

White mice of an inbred Swiss strain were used and after weaning were maintained on stock ration¹ and water *ad libitum* plus occasional greens. With experimental conditions as previously outlined (2, 3), a tissue homogenate was prepared in redistilled water by means of the device described by Potter and Elvehjem (4). From this homogenate a cell-free preparation (1) was obtained which was used immediately in order to minimize inactivation of the enzymes occurring in the very dilute solutions of protein

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¹ B-B Laboratory Rabbit Diet, Maritime Milling Company, Inc., Buffalo, New York.

(5). The dry weights of samples were determined by evaporating to constant weight in open crucibles in an electric oven at 110°.

A conventional Warburg constant volume respirometer at 37.0° was used in all experimental and analytical work and the pH was determined with the Beckman pH meter (glass electrode). Enzyme activities were determined in all cases by measuring the oxygen uptake.

The activity of the succinic oxidase system was determined by the method of Schneider and Potter (6) in which the components of the final reaction mixture (pH 7.6) are as follows: 0.4 ml. of 0.25 M sodium phosphate buffer (pH 7.4), 0.2 ml. of 2×10^{-4} M cytochrome c, 0.3 ml. of 0.5 M sodium succinate (pH 7.4), 0.1 ml. of 0.012 M CaCl_2 , 0.1 ml. of 0.012 M AlCl_3 , 0.2 ml. of 1 per cent cell-free preparation of mouse kidney, solution of inhibitor, and glass-redistilled water to yield a total volume of 3.0 ml. The gas phase was air and 0.2 ml. of 10 per cent NaOH and a small strip of filter paper were placed in the center well to absorb CO_2 .

Cytochrome oxidase was determined by observing the rate of enzymatic oxidation of ascorbic acid as described by Schneider and Potter (6) in which the components of the final reaction mixture (pH 7.6) are as follows: 0.4 ml. of 0.25 M sodium phosphate buffer (pH 7.4), 0.5 ml. of 2×10^{-4} M cytochrome c, 0.3 ml. of 0.114 M ascorbic acid neutralized to pH 7.4 with NaOH, 0.1 ml. of 0.012 M AlCl_3 , the desired amount of 0.67 per cent cell-free preparation, solution of inhibitor, and glass-redistilled water to yield a total volume of 3.0 ml. The gas phase was air and 0.2 ml. of 10 per cent NaOH and a small strip of filter paper were placed in the center well to absorb CO_2 . Two levels of tissue were tested in all cases. The maximum oxygen uptake per hour was plotted for each level and extrapolated to zero concentration in order to correct for the autoxidation of ascorbic acid. Whenever sodium cyanide was used in the above systems, a mixture of alkali and cyanide was used in the center well (7).

The activity of the glutathione oxidase system was determined by a modification² of the method of Ames and Elvehjem (1) in which the components of the final reaction mixture (pH 7.6) are as follows: 0.4 ml. of 0.25 M sodium phosphate buffer (pH 7.4), 0.2 ml. of 2×10^{-4} M cytochrome c, 10 mg. of reduced glutathione, 0.2 ml. of 1 per cent cell-free preparation, solution of inhibitor, and glass-redistilled water to yield a final volume of 3.0 ml. The gas phase was air, and in all cases a correction for the autoxidation of glutathione was made. The rate of autoxidation of glutathione was determined under the same conditions but the tissue and inhibitor were omitted.

Commercially prepared compounds were used without further purifica-

² Ames, S. R., and Elvehjem, C. A., *Arch. Biochem.*, in press.

tion as follows: c.p. $\text{Na}_2\text{HPO}_4 + 12\text{H}_2\text{O}$; c.p. $\text{NaH}_2\text{PO}_4 + \text{H}_2\text{O}$; sodium succinate, Merck; U. S. P. ascorbic acid, Merck; c.p. CaCl_2 ; c.p. AlCl_3 ;

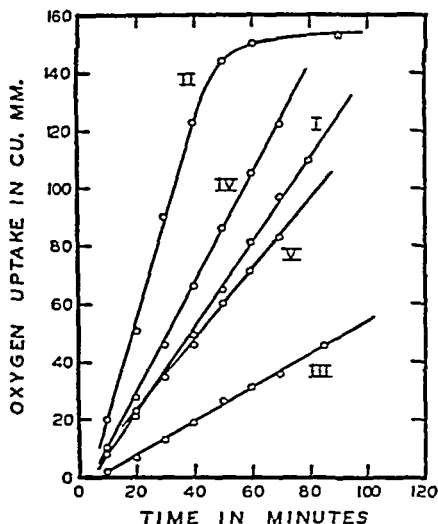


Fig. 1. Typical oxidation of succinate, glutathione, and ascorbate in the presence of cytochrome *c*. Curve I, succinate + 0.2 ml. of 1 per cent cell-free preparation, average rate of 88 c.mm. per hour; Curve II, glutathione + 0.2 ml. of 1 per cent cell-free preparation, average rate of 206 c.mm. per hour; Curve III, glutathione autoxidation (no tissue), average rate of 35 c.mm. per hour; Curve IV, ascorbate + 0.1 ml. of 0.67 per cent cell-free preparation, average rate of 114 c.mm. per hour; Curve V, ascorbate + 0.05 ml. of 0.67 per cent cell-free preparation, average rate of 74 c.mm. per hour. The typical, corrected, maximum Q_{O_2} values calculated from the above data for the oxidations are as follows: succinate, 210; glutathione, 330; and ascorbate, 680. The cell-free preparation was obtained as described in the text. Final concentrations of the components of the reaction mixture of the succinic oxidase system are as follows: 0.033 *M* sodium phosphate buffer, 0.13×10^{-4} *M* cytochrome *c*, 5×10^{-2} *M* sodium succinate, 4×10^{-4} *M* CaCl_2 , 4×10^{-4} *M* AlCl_3 , and 0.067 per cent cell-free preparation (wet weight basis). The final concentrations of the components of the reaction mixture of the glutathione oxidase system are as follows: 0.033 *M* sodium phosphate buffer, 0.13×10^{-4} *M* cytochrome *c*, 0.9×10^{-2} *M* reduced glutathione, and 0.067 per cent cell-free preparation (wet weight basis). The final concentrations of the components of the reaction mixture of the system which oxidizes ascorbate are as follows: 0.033 *M* sodium phosphate buffer, 0.33×10^{-4} *M* cytochrome *c*, 4×10^{-4} *M* AlCl_3 , 1.2×10^{-2} *M* ascorbate, and cell-free preparation at the level of 0.022 per cent and 0.011 per cent, respectively (wet weight basis).

pure, crystalline, reduced glutathione, Eastman Kodak and B. L. Lemke; sodium cyanide, Merck; *p*-nitrophenol, Eastman; sodium arsenite, Malinckrodt; c.p. sodium pyrophosphate, Baker's; 2,4-dinitrophenol, East-

man; 8-hydroxyquinoline, Eastman; sodium diethyldithiocarbamate, Eastman; α, α' -dipyridyl, Eastman; hydroxylamine hydrochloride, Eastman; iodoacetic acid, Eastman; and sodium azide, Eimer and Amend. Cytochrome *c* was prepared in these laboratories from beef heart by a modification³ of the method of Keilin and Hartree (9) and had been dialyzed against glass-redistilled water.

All solutions were prepared in glass-redistilled water and neutralized if necessary to pH 7.4 with dilute NaOH. Glutathione and ascorbic acid solutions were neutralized just before addition to the side arm of the reaction vessel. Substrates were always added from the side arm after a 20 minute period to insure a uniform time of contact between enzyme and inhibitor and to permit the flask contents to reach equilibrium.

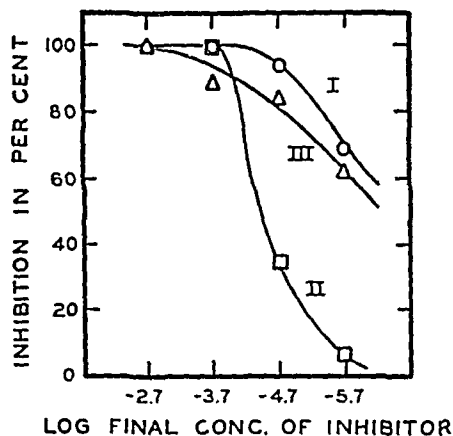


FIG. 2. Effect of cyanide on the extent of inhibition. Curve I, O, succinate; Curve II, □, glutathione; Curve III, Δ, ascorbate. The components of the above system are given in the text and in the legend of Fig. 1. Each experimental point represents the average of several determinations.

The initial substrates which were used as the hydrogen donors for cytochrome *c* were arbitrarily selected as succinate, reduced glutathione, and ascorbate. Fig. 1 illustrates the typical oxidation curves obtained in the absence of any inhibitor. All curves were essentially linear except for the latter portion of the oxidation curve of glutathione, in which the substrate was exhausted after about 50 minutes. In the presence of an inhibitor, the slopes of the curves were decreased, depending upon the concentration of added compound, but the curves were still linear.

In order to compare the action of each of the inhibitors on these three systems the per cent inhibition was plotted against the logarithm of the

³ The method is essentially that given by Potter (8), except that in the first and second centrifugations a Sharples supercentrifuge was used.

concentration of the inhibitor. In the calculation of the per cent inhibition the maximum Q_0 values were used. Examples of this treatment are

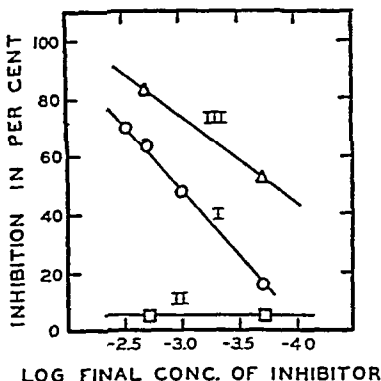


FIG. 3. Effect of azide on the extent of inhibition. Curve I, O, succinate; Curve II, \square , glutathione; Curve III, Δ , ascorbate. The components of the above systems are given in the text and in the legend of Fig. 1. Each experimental point represents the average of several determinations.

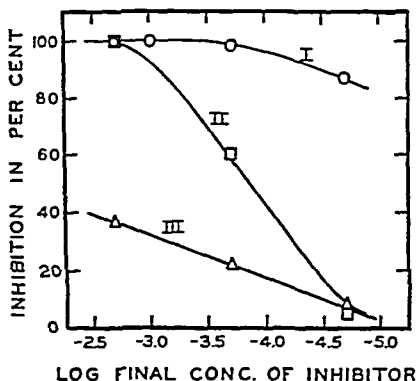


FIG. 4. Effect of diethyldithiocarbamate on the extent of inhibition. Curve I, O, succinate; Curve II, \square , glutathione; Curve III, Δ , ascorbate. The components of the above systems are given in the text and in the legend of Fig. 1. Each experimental point represents the average of several determinations.

given for cyanide, azide, and diethyldithiocarbamate in Figs. 2, 3, and 4, respectively. The action of cyanide was stronger than that of the other inhibitors, being still effective at concentrations of about 10^{-5} M. All

three systems were inhibited to essentially the same degree by cyanide; at low concentrations only, the glutathione oxidase system showed less inhibition than the other two systems. Azide exhibited marked inhibition of the oxidations of succinate and ascorbate but had essentially no effect on the oxidation of glutathione even in concentrations as high as 0.002 M. This is in marked contrast with the type of inhibition obtained in the presence of diethyldithiocarbamate. Under the influence of this inhibitor,

TABLE I

Effect of Other Inhibitors on Oxidation of Succinate, Glutathione, and Ascorbate

Inhibitor			Per cent of inhibition		
Compound	Type	Final concentration $\times 10^4$	Succinic oxidase	Glutathione oxidase	Enzymatic oxidation of ascorbate
		M			
8-Hydroxyquinoline.....	Cu	0.14	5	39	N.e.
Na thioglycolate.....	Fe	2	25	11	18
Hydroxylamine.....	"	2	61	9	40
Na pyrophosphate.....	"	2	43	S.	S.
α, α' -Dipyridyl.....	"	2	37	15	N.e.
Na iodoacetate.....	—SH	2	48	64	45
" arsenite.....		2	63	S.	59
2,4-Dinitrophenol.....		0.041	5	"	S.
p-Nitrophenol.....		2	13	0	"

The components of the above systems are as described in the text and in the legend of Fig. 1. In the calculation of the per cent of inhibition, the maximum Q_{O_2} values were used after a 20 minute contact time between the inhibitor and enzyme. Most experimental values represent the average of several determinations. S. denotes that a Q_{O_2} greater than that of the control flask was observed, indicating that the addition of that compound resulted in a slight increase in the oxygen uptake. N.e. denotes that a non-enzymatic reaction occurred in which the rapid oxygen uptake masked that of the enzymatic reaction and that no inhibition value could be obtained.

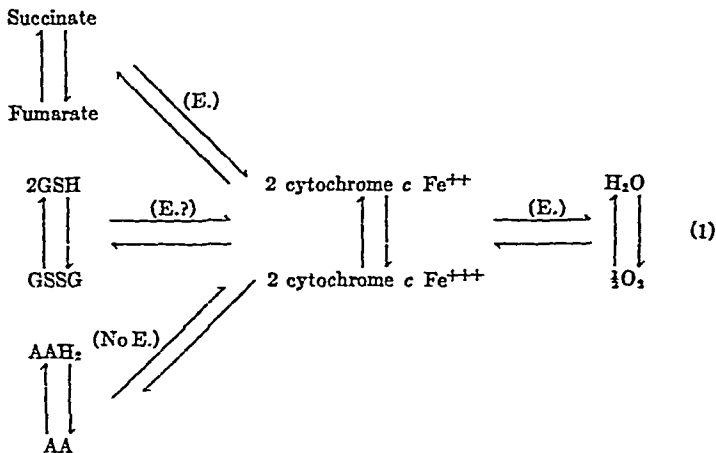
the oxidation of ascorbate shows low inhibitions in the ranges in which the oxidations of both succinate and glutathione are almost entirely inhibited.

A number of other compounds which might act as inhibitors in these systems were tested and the results are summarized in Table I along with a consideration of the general type of inhibition which that compound exhibited previously. The extent of inhibition at only one concentration is given for each of the inhibitors in Table I, but this is sufficient for comparative purposes. As an example of a typical copper inhibitor, 8-hydroxyquinoline is similar to diethyldithiocarbamate in its effect on oxygen uptake, with the exception of its being involved in a chemical reaction with ascorbic acid. In a similar manner, the action of several compounds

forming complexes with iron essentially parallels that of azide in inhibitory characteristics. In some cases an increase in observed oxygen uptake was obtained on addition of the compound, and in several cases non-enzymatic reactions were noted, effectively masking observation of any enzymatic reaction which might be taking place. Where values are given for the degree of inhibition, no evidence for the occurrence of non-enzymatic reactions between inhibitors and initial substrates has been observed.

DISCUSSION

The systems under investigation might be represented in a simple schematic form (Equation 1) where GSH and GSSG represent reduced



and oxidized glutathione, respectively, AAH₂ and AA represent reduced and oxidized ascorbate, respectively, and E. indicates that the reaction is enzymatically catalyzed. A dehydrogenase, analogous to that which functions in the oxidation of succinate (10), is postulated on the basis of preliminary evidence for the glutathione oxidase system (1), and no enzyme has been proposed for the oxidation of ascorbate by oxidized cytochrome *c*. The enzyme functioning between cytochrome *c* and molecular oxygen is by definition cytochrome oxidase (11), the properties of which have been reviewed recently by Stotz (12). Cytochrome oxidase is believed to be identical (13) with the *Atmungsferment* which was indicated to be a hemin-containing compound (14). Considerable indirect evidence has been presented to indicate that a copper-protein is involved in the oxidation of cytochrome *c* (15). Keilin and Hartree (16) have summarized supporting evidence for both the copper-protein and iron-protein hypotheses.

The inhibition of the cytochrome oxidase system by azide and cyanide has been reported (17, 18) but the enzyme was not inhibited by sulfhydryl reagents (19). In the succinic oxidase system the dehydrogenase itself was reported to be unaffected by cyanide and pyrophosphate (10), but another investigation (20) indicated that pyrophosphate was strongly inhibitory. Succinic oxidase was reported to be inhibited by cyanide and arsenite (21), diethyldithiocarbamate (22), and also iodoacetate (19). The glutathione oxidase system has been previously reported (1) to be strongly inhibited by cyanide, diethyldithiocarbamate, and iodoacetate, weakly inhibited by α, α' -dipyridyl and thioglycolate, and not inhibited by azide and hydroxylamine.

A number of metallo proteins have been previously studied by observing the inhibition obtained on using the three compounds considered in detail. Azide has been shown to inhibit catalase (Fe) (23), peroxidase (Fe) (24), cytochrome oxidase (Fe?) (17), and carbonic anhydrase (Zn) (25). Diethyldithiocarbamate has been reported to inhibit tyrosinase (Cu) (26), laccase (Cu) (27), and ascorbic acid oxidase (Cu) (28), in addition to being a standard reagent in copper determinations (29). An oxidation product of diethyldithiocarbamate has been reported to inhibit succinic oxidase by acting as a sulfhydryl inhibitor (22). Cyanide is considered to be a general inhibitor of both iron- and copper-containing enzymes. In general, azide is an inhibitor for iron-containing enzymes, diethyldithiocarbamate inhibits copper-containing enzymes, and cyanide inhibits both. Other less common trace metals may form complexes with proteins which might be inhibited in a similar manner but, until the enzyme in question is isolated and analyzed, no conclusions along this line are justified.

If the hydrogen donor-cytochrome *c*-cytochrome oxidase-oxygen system is as simple as is diagrammed in Equation 1, with cytochrome oxidase considered to be a single metal enzyme and the dehydrogenases considered as sulfhydryl-containing enzymes, the inhibition data should be the same, independent of the hydrogen donor. This is not the case when the hydrogen donors are succinate, glutathione, and ascorbate. Glutathione appears to be oxidized by a system containing copper as an integral part, in contrast with ascorbate, the oxidation of which is depressed by iron inhibitors. The oxidation of succinate involves both types of inhibition, one resembling that exhibited by a copper-containing enzyme and the other an iron-containing enzyme. These data indicate that the oxidation of the three compounds is not as simple as is diagrammed in Equation 1 and show not only that copper or a metal resembling it may be involved in addition to iron but that, by changing the hydrogen donor, different mechanisms may be employed. The possibility may be indicated that the systems reacting

between the initial hydrogen donors and cytochrome *c* involve a metallo protein.

SUMMARY

1. The oxidation of succinate, reduced glutathione, and ascorbate by molecular oxygen with cell-free tissue preparations and added cytochrome *c* was observed, and the effects of the following inhibitors were investigated: cyanide, azide, diethyldithiocarbamate, 8-hydroxyquinoline, thioglycolate, hydroxylamine, pyrophosphate, α, α' -dipyridyl, iodoacetate, arsenite, 2,4-dinitrophenol, and *p*-nitrophenol.

2. By altering the initial hydrogen donor in the system, hydrogen donor-cytochrome *c*-cytochrome oxidase-oxygen, the inhibition properties are changed and appear to involve different metallo proteins as follows: reduced glutathione, copper-containing enzyme; ascorbate, iron-containing enzyme; and succinate, both iron- and copper-containing enzymes.

3. The above system appears to be more complex than commonly represented, involving at least two separate types of inhibition, the properties of which resemble those attributed to iron- and copper-containing enzymes.

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FLUOROMETRIC STUDIES OF THE RIBOFLAVIN CONTENTS OF MUSCLE AND LIVER

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With due acknowledgment to the previous workers in this field, it seems unnecessary to ask space here for a full review of the individual contributions that have established the general distribution of riboflavin in the tissues. And any attempt at precise quantitative comparisons of past findings might be misleading inasmuch as methods were still in course of development.

The fact that riboflavin exists in the body to an important degree as an oxidation enzyme in the tissues and the question of the body's ability to store riboflavin have led us to study quantitatively the riboflavin contents of muscle and liver in normal rats of different ages and after feeding at different levels of riboflavin content of food.

EXPERIMENTAL

Riboflavin was here determined by fluorometric measurement by a method developed from that described by Hodson and Norris (1).

From 0.3 to 3.5 gm. of tissue, depending on riboflavin content, were macerated and extracted with 50 ml. of 0.25 N sulfuric acid for 30 minutes in a boiling water bath. After cooling, the suspension was adjusted to pH 5.0 to 6.0, made up to volume, and filtered. Fluorescence measurements were made on (a) an aliquot of the filtrate diluted with an equal volume of water and (b) on a similar aliquot of filtrate to which had been added the same volume of standard riboflavin solution. A blank was obtained by the addition of sodium hydrosulfite solution; this reading was consistently low in these analyses.

Experiments showed that riboflavin added at the beginning of the extraction was completely recovered.

In determinations on blood, sodium sulfate was added at the conclusion of the extraction period to precipitate hemoglobin. The filtrate was pale yellow in color; because of this and the low riboflavin concentration of blood, these data are less precise than those for muscle and liver.

Values for riboflavin obtained by this method were in good agreement with those found by the method of Van Duyne (2), in which the extraction of riboflavin was effected by enzymatic hydrolysis with pepsin. Rosner,

Lerner, and Cannon (3) similarly report uniform values, in which the hydro-sulfite blank is low and adsorption techniques are omitted, between acid-extracted samples and samples treated both with acid and the enzyme polidase.

The experiments were made upon normal rats from the laboratory-bred colony of this Department. The animals were all closely related genetically and were of like nutritional background, except that half came from families whose food (Diet 485) contained twice as much riboflavin as that of the others (Diet 16). This Diet 16, also called Diet A in some of the

TABLE I

Riboflavin in Normal Rat Muscle

Riboflavin is expressed in micrograms per 100 gm.

	From Diet 16		From Diet 485	
	Males	Females	Males	Females
30 days old, mean.....	413	420	426	428
P. e.* of mean.....	± 3.7	± 4.3	± 4.2	± 5.7
Coefficient of variation.....	5.8	5.7	6.2	8.3
No. of cases.....	18	14	17	17
60 days old, mean.....	342	356	357	368
P. e. of mean.....	± 7.0	± 4.9	± 10.1	± 6.3
Coefficient of variation.....	8.6	6.9	10.4	6.4
No. of cases.....	8	11	6	6
360-500 days old, mean.....	238	219		
P. e. of mean.....	± 12.7	± 10.2		
Coefficient of variation.....	21.0	19.6		
No. of cases.....	7	8		
Between 30 and 60 days, difference.....	71	64	69	60
P. e. of difference.....	± 7.9	± 6.5	± 10.9	± 8.5
Critical ratio.....	9.0	9.8	6.3	6.1
Between 60 and 360-500 days, difference....	104	137		
P. e. of difference.....	± 14.5	± 11.3		
Critical ratio.....	7.2	12.1		

* This is the classical probable error of the mean.

papers from this laboratory, is an air-dry mixture of five-sixths ground whole wheat and one-sixth dried whole milk, fed with sodium chloride in the proportion of 2 per cent of the weight of the wheat, and with distilled water *ad libitum*. Diet 485 contained the same ingredients as Diet 16 with the addition of pure riboflavin sufficient to increase its riboflavin content to 6 to 7 γ per gm. of air-dry food mixture, or twice the riboflavin content of Diet 16 which contains 3 to 3.5 γ of riboflavin per gm. of dry food.

Special attention was given to care and uniformity of procedure in the killing of the animals and the removal and handling of the large leg muscle and the liver for analysis.

DISCUSSION

The data obtained in our main series of analyses are summarized in Tables I and II.

For economy of space, the findings are given in terms of mean values with the number of cases entering into each mean, and (where the numbers warrant) the probable error of each mean and the coefficient of variation. As all details of method and operation were uniform throughout the experiments and analyses reported in this paper, it is probable that the coeffi-

TABLE II
Riboflavin in Normal Rat Livers

Riboflavin is expressed in micrograms per 100 gm.

	From Diet 16		From Diet 485	
	Males	Females	Males	Females
30 days old, mean	2650	2540	2580	2840
P. e. * of mean	±48	±68	±54	±72
Coefficient of variation	11.5	13.4	12.6	15.1
No. of cases	18	11	16	16
60 days old, mean	2814	3079	3293	3192
P. e. of mean	±63.0	±50	±163	±95
Coefficient of variation	9.4	8.0	18.0	10.8
No. of cases	8	11	6	6
360-500 days old, mean	3707	2801		
P. e. of mean	±68	±38		
Coefficient of variation	7.2	5.7		
No. of cases	7	8		
Between 30 and 60 days, difference	164	539	713	352
P. e. of difference	±79.2	±84.3	±171.7	±119.3
Critical ratio	2.1	6.4	4.2	3.0
Between 60 and 360-500 days, difference	893	-278		
P. e. of difference	±92.8	±62.7		
Critical ratio	9.6	4.4		

* This is the classical probable error of the mean.

icients of variation here found are fairly indicative of the true physiological variations among animals of like genetic and nutritional backgrounds. Data for muscle are given in Table I, and those for liver in Table II.

Both in muscle and in liver the mean riboflavin contents found at a given age were essentially alike for the animals from the basal Diet 16 (with 3 to 3.5 γ of riboflavin per gm. of air-dry food) and from Diet 485, which contained twice as much riboflavin. Thus an intake level of 3 to 3.5 γ of riboflavin per gm. of air-dry food, or 0.8 to 0.9 γ of riboflavin per kilocalorie of food, appears to have been sufficient for the support of "plateau values" of

riboflavin content of muscle and liver, these values not being significantly increased by a doubling of the riboflavin content of the food. Also, no significant difference between the sexes was found.

There is, however, a slight decrease in the average riboflavin content of the muscle at 60 days from that found at 30 days of age. Our analyses of the muscles of rats 360 to 500 days old, while too few to be conclusive, clearly suggest a further decrease in the riboflavin content of muscle between adolescence and middle age.

At 30 days of age our rats contained 6.0 to 6.6 times as much riboflavin per gm. of tissue in liver as in muscle. At 60 days of age, the concentration was 8.2 to 9.2 times as high in liver as in muscle, due to a regular (and statistically significant) decrease in muscle at the same time with an irregular increase in the liver.

Our findings for the riboflavin contents of the blood (of the same animals just mentioned) were of the order of 0.1 to 0.2 γ per gm. Because we regard these data as less precise we omit any detailed tabulation or statistical treatment of them.

SUMMARY

In normal rats of both sexes at 30 days of age ("end of infancy") and from diets containing either 3 to 3.5 γ of riboflavin per gm. of air-dry food or twice this level, the concentration of riboflavin in muscle was fairly constant, averaging 4.13 to 4.28 γ per gm. Between the ages of 30 and 60 days, the riboflavin concentration in muscle underwent a statistically significant diminution; and further diminution with age is indicated by the few cases here examined at ages of 360 to 500 days. This change of concentration in muscle with age is being studied further.

Livers of 30 day- or 60 day-old rats showed riboflavin concentration more variable than that of muscle and 6- to 9-fold higher. Blood, studied in fewer cases, showed still greater variability, with an average riboflavin concentration much lower than that of muscle and liver.

The aid of grants from the Carnegie Institution of Washington is gratefully acknowledged.

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A NOTE ON THE CONVERSION IN VIVO OF THE S-BENZYL-N-METHYL DERIVATIVES OF CYSTEINE AND HOMOCYSTEINE TO THE N-ACETYL-S-BENZYL DERIVATIVES OF CYSTEINE AND HOMOCYSTEINE

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Some time ago in discussions on N-methylmethionine (1), N,N'-dimethylhomocystine (1), and N,N'-dimethylcystine (2), the argument was advanced that demonstration of the growth-promoting power of these methylated amino acids was tantamount to demonstrating that their keto acid analogues were also capable of supporting growth. It was thus inferred that the α -keto acids derivable from homocystine, methionine, and cystine were utilizable for growth purposes. Since that time, the keto acid has been found to be effective in supporting the growth of rats on a diet deficient in methionine (3).

It is of interest that *d*-amino acid oxidase and broken cell preparations of rat kidney and liver have been shown to convert *dl*-N-methylmethionine to the γ -methylmercapto- α -ketobutyric acid (4). Furthermore, the *d* forms of S-benzylhomocystine and S-benzylcystine have been found to be converted *in vivo* to their corresponding N-acetyl-S-benzyl-*l* derivatives and excreted as such in the urine (5). In the conversion of a *d*-amino acid to its acetyl-*l* derivative, the keto acid has been considered a likely intermediate followed by asymmetric synthesis of the *l*-acetyl compound (6). It occurred to us, therefore, that we had the opportunity of testing whether the intact animal is capable of carrying out the sequence of reactions in keeping with the above theory for the utilization of N-methyl-amino acids.

It is evident that if the N-methyl derivatives of S-benzylhomocystine and S-benzylcystine are oxidatively deaminized to the keto acid, then one should be able to isolate the N-acetyl-S-benzyl-*l*-homocystine and N-acetyl-S-benzyl-*l*-cystine, respectively, from the urines after the corresponding N-methyl derivatives were fed. This was found to be the case in the experiments we carried out.

N-Methyl-S-benzyl-*l*-cystine was prepared by the method previously described (2). The compound had a rotation of $[\alpha]_D^{25} = +64^\circ$ for a 1 per cent solution in 1 N HCl, and melted at 207–208°. 1.5 gm. of N-methyl-S-benzyl-*l*-cystine admixed with the food were given to a white

rat. The basal diet and the procedure for the isolation of the products from the urine were the same as those previously described (5). 70 mg. of long rod-like crystals were isolated from the urine. These crystals melted at 142–143° and the melting point of a mixture of this product with synthetic N-acetyl-S-benzyl-L-cysteine, m.p. 143–144°, $[\alpha]_D^{25} = -42^\circ$ (5), was the same. The product obtained had a rotation of $[\alpha]_D^{25} = -46.5^\circ$ for a 1 per cent solution in 95 per cent ethanol. The nitrogen value checked the theoretical value of 5.5 per cent.

The N-methyl-S-benzyl-DL-homocysteine was prepared as previously described (1). 2 gm. of N-methyl-S-benzyl-DL-homocysteine were fed to a white rat. 27 mg. of crystals were isolated from the urine (5). This material had a specific rotation of $[\alpha]_D^{25} = +5^\circ$ in 5 cc. of 95 per cent ethanol. The melting point was 131.5° and the compound produced no depression in the melting point when mixed with an authentic sample of N-acetyl-S-benzyl-L-homocysteine, m.p. 131–132°, $[\alpha]_D^{25} = +5.5^\circ$ (5). The analysis of the isolated product showed a nitrogen value of 5.1 per cent, agreeing with the theoretical value of 5.24 per cent.

SUMMARY

N-Acetyl-S-benzyl-L-homocysteine has been isolated from the urine of a rat fed N-methyl-S-benzyl-DL-homocysteine, and N-acetyl-S-benzyl-L-cysteine has been isolated from the urine of a rat fed N-methyl-S-benzyl-L-cysteine.

The possible relationship of these findings to the metabolism of N-methylamino acids and the corresponding keto acids has been discussed.

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PHOSPHOGLYCOCYAMINE

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Glycocyamine has received special attention in recent years on account of the rôle it plays as an intermediate in the biosynthesis of creatine. It has long been known as a body constituent and more recently methods have been developed for its estimation in biological material (1). The question naturally arises as to whether tissue glycocyamine, like its higher homologue creatine, exists partly in the phosphorylated form, or whether phosphorylation of glycocyamine is an essential step in the biological synthesis of creatine or phosphocreatine. These questions cannot be answered before phosphoglycocyamine itself has been prepared and a study made of its chemical and biological properties.

EXPERIMENTAL

Preparation of Phosphoglycocyamine—The method is essentially that employed in the preparation of the related compounds, phosphocreatine and phosphoguanidine (2, 3); viz., phosphorylation with 3 moles of POCl_3 in a strongly alkaline aqueous medium, removal of the excess glycocyamine by evacuation of the neutralized solution, precipitation of inorganic phosphate first by magnesia mixture, then with calcium chloride-calcium hydroxide, and precipitation of the calcium salt of phosphoglycocyamine by the addition of 2 volumes of alcohol. The calcium salt is then purified as follows: centrifuge, remove sodium chloride by repeated suspension of the precipitate in a little water, followed by precipitation with 2 volumes of alcohol. The precipitate is then dissolved by repeated extractions (shaking machine) with water, and evacuation of the extracts to a small volume by use of the ice-desiccator method (4). The calcium salt that crystallizes out is centrifuged, washed with 70 per cent alcohol, then with 95 per cent alcohol, and finally with ether. From 5 gm. of glycocyamine 1.8 gm. of phosphoglycocyamine-calcium salt are obtained which is sufficiently pure for enzymatic studies.

To obtain an analytically pure product 1 gm. of the salt is suspended in 50 cc. of ice-cold water and dissolved by the addition of a few drops of dilute hydrochloric acid. Neutralize immediately to phenolphthalein

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with powdered calcium hydroxide, filter, and add 3 cc. of 50 per cent calcium chloride and 2 volumes of alcohol. A crystalline precipitate (spherulites) is formed which, after being washed with alcohol and dried, is redissolved and neutralized with calcium hydroxide as described. The filtrate is now made acidic to brom-cresol purple before precipitation with alcohol as in the Fiske and Subbarow procedure for the purification of phosphocreatine (5). The washed precipitate is air-dried and analyzed.

$C_7H_{10}O_7N_2PCa \cdot 3H_2O$	Calculated.	C 12.4,	H 4.1,	N 14.5,	P 10.7
	Found.	" 12.95,	" 4.05,	" 13.8,	" 10.4

Hydrolysis of Phosphoglycocyamine in Acid Solution—Phosphoglycocyamine is distinctly more stable than phosphocreatine; whereas phosphocreatine is completely hydrolyzed in 30 minutes, if allowed to stand at room temperature in Fiske and Subbarow's acid molybdate reagent (5), phosphoglycocyamine is hydrolyzed only to the extent of 15 per cent at 28° under these conditions. After 4 hours 55.7 per cent and after 10 hours 87 per cent hydrolysis takes place.

In normal hydrochloric acid at 100° phosphoglycocyamine is hydrolyzed completely in 2 minutes. It is thus more labile than the two "hydrolyzable" phosphate groups in adenosine triphosphate which under the same conditions are split in 7 minutes. Phosphoglycocyamine can thus be determined in the presence of adenosine triphosphate, as will be shown below.

Enzymatic Studies

Transfer of Phosphate from Phosphoglycocyamine to Adenylic Acid with Formation of Adenosine Triphosphate—In the presence of Meyerhof's enzyme from rabbit muscle extract and under the conditions set forth by Lohmann (6), phosphoglycocyamine transfers its phosphate to adenylic acid. In this respect phosphoglycocyamine is slightly less active than phosphocreatine, mole for mole. Table I which represents an average of several experiments illustrates this fact. Phosphoguanidine, on the other hand, does not transfer its phosphate to adenylic acid.

Methods

Phosphorus Compounds—Adenylic acid and adenosine triphosphate were prepared according to Kerr (7, 8); phosphocreatine and phosphoguanidine as mentioned above (2, 3).

Enzyme—Meyerhof's enzyme was prepared from rabbit muscle by extracting 2 parts of minced muscle with 3 parts of ice-cold water. The extract was dialyzed for 1 week in the ice chest against distilled water and 0.5 per cent KCl alternately. To this extract bicarbonate and phosphate

were added according to Lohmann (6) and the pH brought to 7.2 to 7.3 by bubbling CO_2 through it.

Determination of Phosphorus Compounds—Inorganic phosphate plus phosphocreatine phosphorus was determined by the method of Fiske and Subbarow (5). The "hydrolyzable" phosphorus of adenosine triphosphate was taken as the amount of inorganic phosphate formed after heating for 7 minutes in N HCl at 100° . The amount of adenosine triphosphate formed by the action of phosphoglycocysteamine on adenylic acid is determined indirectly in the following manner. After incubation with the enzyme and precipitation of the enzyme protein by adding an equal volume

TABLE I

Transfer of Phosphate from Phosphoglycocysteamine to Muscle Adenylic Acid

In each case 1 cc. of Lohmann's enzyme mixture (6) containing 0.44 mg. of inorganic P was used. To this the solutions containing the phosphorus compounds were added and the final volume was made up to 4 cc. by the addition of water. Incubation was for 1 hour at 28° , after which precipitation with 4 cc. of 10 per cent trichloroacetic acid was carried out. The figures represent mg. of phosphorus.

	Phosphocreatine + inorganic phosphate	2 min. hydrolysis value	7 min. hydrolysis value	Hydrolyzable P formed
Phosphocreatine, 0.44 mg. P + adenylic acid, 0.223 mg. P	0.46	0.74	0.83	0.37
Phosphoglycocysteamine, 0.45 mg. P + adenylic acid, 0.223 mg. P	0.49	0.79	0.88	0.30
Phosphoglycocysteamine, 0.45 mg. P + enzyme	0.55	0.87	0.87	0.00
Phosphoguanidine, 0.62 mg. P + adenylic acid, 0.223 mg. P	0.51	1.03	1.03	0.00

of 10 per cent trichloroacetic acid, aliquot samples were taken from each specimen and HCl added to make the solution 1 N . The tubes are immersed in a briskly boiling water bath and a 2 minute and a 7 minute hydrolysis value obtained. These values are the same in the control tubes, *i.e.* when only phosphoglycocysteamine was added to the enzyme solution, but are different when adenosine triphosphate is formed. The percentage hydrolysis of adenosine triphosphate after 2 minutes is determined more accurately on the adenosine triphosphate formed enzymatically from phosphocreatine and adenylic acid than from a control containing adenosine triphosphate only. Under the conditions of the experiments represented in Table I, an average of 73 per cent of the "hydrolyzable" phosphorus of adenosine triphosphate was split in the first 2 min-

utes and 27 per cent in the last 5 minutes. The "hydrolyzable" phosphate formed from phosphoglycocyamine would thus be the difference between the 2 and 7 minute values divided by 0.27. The 2 minute value varies from one experiment to the other, depending on the time it takes the solution in the immersed tubes to reach 100° and on the rate of cooling after the 2 minutes are over. It is therefore essential that all specimens be hydrolyzed at one time and in test-tubes of the same size and quality.

Other Enzymatic Studies—The reaction phosphocreatine + adenylic acid = adenosine triphosphate + creatine is reversible (6), the reverse reaction being favored in alkaline medium (9). We have tried to ascertain whether the reverse reaction would take place with glycocyamine instead of creatine but no phosphoglycocyamine was formed in neutral or alkaline (pH 10) medium, as was evidenced by the fact that the 2 and 7 minute hydrolysis values in the test experiment were the same as in the control containing only enzyme and adenosine triphosphate. In this connection it must be mentioned that in our own hands and by the use of our enzyme preparations the reverse reaction, *i.e.* the formation of phosphocreatine from creatine and adenosine triphosphate, could be made to take place only at pH 10; at pH 7.2 and under the same conditions that the forward reaction proceeded no trace of phosphocreatine was formed. Finally, it was found that neither at pH 7.2 nor at pH 10 was there an enzymatic transfer of phosphate from phosphocreatine to glycocyamine or from phosphoglycocyamine to creatine.

DISCUSSION

It is apparent from the results given above that the reaction phosphoglycocyamine + adenylic acid = adenosine triphosphate + glycocyamine takes place *in vitro*. Whether and to what extent this reaction plays a significant rôle *in vivo* is at present a matter of speculation. There is no reliable information on the glycocyamine content of muscle tissue. Borsook and Dubnoff (10) give a figure of 3 to 6 mg. per cent. By their method, however, one would not be expected to extract all the glycocyamine if the latter existed partly in the phosphorylated form. As a labile phosphorus compound, phosphoglycocyamine could easily escape detection and would be included in the figure for "hydrolyzable" phosphorus which represents the two labile phosphate groups of adenosine triphosphate.

SUMMARY

1. The synthesis of phosphoglycocyamine is described.
2. In the presence of Meyerhof's enzyme from rabbit muscle extract phosphoglycocyamine, like phosphocreatine but unlike phosphoguanidine, transfers its phosphate to adenylic acid with the formation of adenosine

triphosphate. This reaction, however, is not reversible either in neutral or in alkaline medium.

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A METHOD FOR THE DETERMINATION OF ALLOXAN

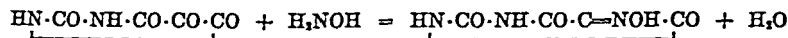
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It was to be expected that the discovery of the diabetogenic action of alloxan would stimulate analysts in a search for methods for the determination of this remarkable compound. Whereas up to 1945 no such method was available, with the exception of a half quantitative semimicro procedure by Lieben and Edel (1), early that year two methods were published by Leech and Bailey (2), no less than six by Archibald (3), and finally a highly sensitive test by Banerjee, Dittmer, and du Vigneaud (4). Leech and Bailey's procedures are of only moderate specificity, and in their present form allow the determination of blood alloxan only, while none of the other methods has yet been adapted to the analysis of alloxan in biological materials, except for some recovery experiments with various blood filtrates (3). The present method, which is comparatively simple, highly specific, and adapted to the determination of alloxan in blood, tissues, and other material, may therefore be of some value.

Alloxan in amounts varying from 0.04 to 0.4 mg. can be determined by the method which is based on the following principle: (a) conversion of alloxan to violuric acid by evaporation *in vacuo* with hydroxylamine, according to



(b) photometric determination of the violuric acid in the form of its ferrous complex, probably according to



EXPERIMENTAL

Color Reaction of Violuric Acid with Bivalent Iron

The blue color resulting from the addition of ferrous salts to solutions of violuric acid seems to have been first observed by von Baeyer (5). In order to develop a method for the estimation of violuric acid, it was decided to investigate the reaction more in detail. Early in this study it became evident that the comparatively weak color could be greatly strengthened by the addition of small amounts of alkali. At the same time the stability

of the solution decreased rapidly, and repeated filtrations became necessary to obtain satisfactory photometric readings. This difficulty was largely overcome by the addition of 20 per cent glycerol, which not only stabilized the color for 10 to 20 minutes over a wide range of pH, but also increased it substantially. Thus it became possible to study the influence of pH and dilution on the color.

To 0.6 mg. of violuric acid were added 2 cc. of 50 per cent glycerol, about 15 mg. of solid ferrous ammonium sulfate, and borate buffers of various pH up to 5 cc. final volume. The extinction which was read in the Pulfrich photometer, with a 0.5 cm. cuvette and Filter 61, rose from 0.52 at pH 7.26 to 1.50 at pH 9.97. At higher pH, the solutions were very unstable and rapidly became turbid, making correct readings impossible. Some experiments with glycine buffer gave similar results.

After reading, the colors were diluted with the respective buffers, to which 20 per cent glycerol had been added, and again read. The extinction values thus obtained decreased quite out of proportion to the dilution, *e.g.* from 1.18 (undiluted, pH 8.9) to 0.44 (1:1 dilution, same pH), or from 1.11 (undiluted, pH 8.57, glycine buffer) to 0.20 (1:3 dilution, same buffer). Better results were not obtained if instead of dilution smaller amounts of violuric acid, 0.15 or 0.3 mg., were determined directly, indicating that Beer's law is not valid under these conditions. In order to test whether this was brought about by dissociation of the colored compound into colorless ions, attempts were made to depress the dissociation by various additions. Glycerol, alcohol, ferrous sulfate, and magnesium sulfate all strongly increased the color, roughly in proportion to the amounts added. Once this had been established, it was decided to carry out the reaction in a water-free solution, in order to prevent dissociation as far as possible; therefore absolute alcohol was chosen as the solvent.

In alcoholic solutions, strong and stable colors were obtained, which did not further increase upon the addition of glycerol or any of the other substances mentioned above. Small amounts of alkali had only a small effect on the color. After dilution with absolute alcohol, the extinction coefficients of the solution decreased in exact proportion to the dilution over a wide range of concentration. A final difficulty arose when it was observed that hydroxylamine hydrochloride, the presence of which is necessary during the later stage of the determination, caused a substantial depression of the color, owing to a decrease in pH from 5.8 in the original solutions to about pH 5.0 after addition of hydroxylamine. This was overcome by the addition of quinine hydrochloride, which is easily soluble in alcohol and stabilizes the pH at 5.9.

A few remarks may be appropriate concerning the composition of the colored reaction product. Kuester (6) isolated a blue compound from aque-

ous solutions of violuric acid and ferrous acetate, which was insoluble in organic solvents and which had an iron content corresponding to a simple iron salt with the formula $\text{Fe}(\text{C}_4\text{H}_2\text{O}_4\text{N}_2)_2$. The possibility of complex salt formation should not be overlooked, however, particularly since Hantzsch (7) isolated a number of complex salts of violuric acid with monovalent metals, with the general formula $\text{MeC}_4\text{H}_2\text{O}_4\text{N}_2 + \text{C}_4\text{H}_2\text{O}_4\text{N}_2$. To obtain some experimental evidence on this point we tried to determine the minimum amount of iron (as ferrous ammonium sulfate), sufficient to give the optimum color with a given amount of violuric acid. On gradual addition, the optimum was reached with 35 γ of iron per 0.3 mg. of violuric acid, and further addition remained without effect. This figure corresponds fairly closely to the 32 γ required by a compound of the complex formula $\text{Fe}(\text{C}_4\text{H}_2\text{O}_4\text{N}_2)_2 + \text{C}_4\text{H}_2\text{O}_4\text{N}_2$. The iron violurate of Kuester would require 45.7 γ of Fe.

Construction of Standard Curve for Photometric Determination of Violuric Acid

60 mg. of violuric acid, prepared either from alloxan and hydroxylamine, or from barbituric acid and sodium nitrite, and dried to constant weight at 100°, were dissolved in 100 cc. of absolute alcohol. This solution remains stable for months, even at room temperature. To various amounts of this standard were added 1 cc. of 50 per cent (by volume) glycerol in absolute alcohol, 10 mg. of solid hydroxylamine hydrochloride, and absolute alcohol up to 5 cc. Cautious heating below the boiling point facilitates solution of the hydroxylamine. 0.2 gm. of quinine hydrochloride was next added, followed by 0.1 cc. of a saturated solution of ferrous ammonium sulfate in 50 per cent glycerol-alcohol. The blue color which develops immediately remains stable for hours.

Extinction was measured in the Pulfrich photometer, with a 1 cm. cuvette and Filter 61 (610 $m\mu$). Fig. 1 shows that Beer's law holds satisfactorily between 0.04 and 0.4 mg. of violuric acid. Above 0.15 mg. the color is so strong that it is better read in a 0.5 cm. cuvette. Fig. 2 demonstrates the spectral distribution of the absorption, as determined roughly with the nine filters available for our instrument.

Conversion of Alloxan into Violuric Acid

Violuric acid is prepared with nearly theoretical yield by mixing equimolecular amounts of alloxan and hydroxylamine in saturated solutions. An attempt was therefore made to evaporate dilute solutions of the two substances *in vacuo*, and very satisfactory yields were obtained when the violuric acid formed was determined by the method outlined above. The conversion became quantitative when alcohol, small amounts of glycerol,

or both, were present during the evaporation. Since the latter substance prevented spattering of the contents of the flask towards the end of the process and possibly stabilized the colors later obtained, its addition was incorporated into the procedure finally adopted. It should be emphasized, however, that the presence of glycerol has no influence on the strength of

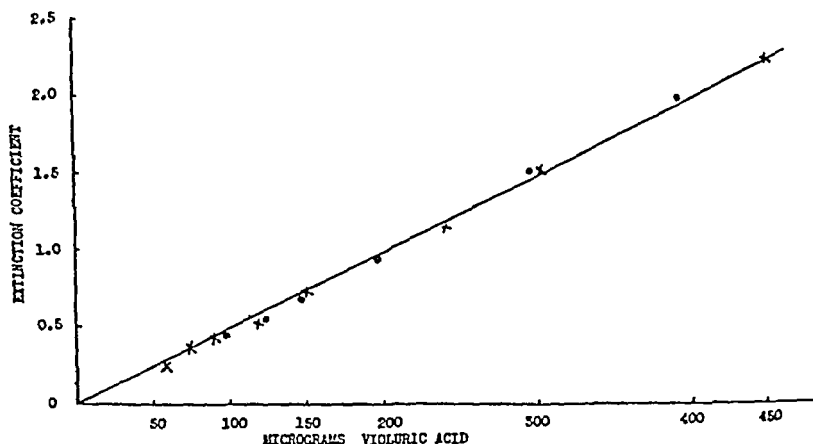


FIG. 1. Standard curve for the determination of violuric acid (X) and recovery of alloxan as violuric acid (O).

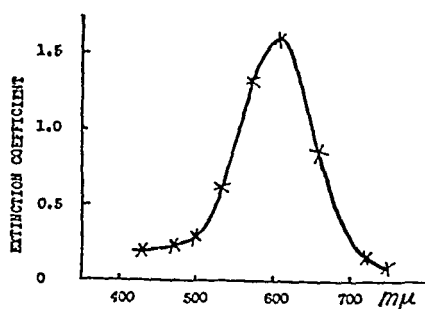


FIG. 2. Spectral distribution of the extinction coefficient of iron violurate (0.3 mg.) in alcoholic solution.

the color of iron violurate in alcoholic solution, and is used only for its physical properties during the evaporation.

An excess of hydroxylamine was found necessary in order to obtain quantitative yields of violuric acid. For amounts up to 0.25 mg. of alloxan, 10 mg. were sufficient, but for 0.4 mg. 18 mg. were needed.

Determination of Alloxan

Apparatus—This is an ordinary 50 cc. distillation flask, fitted with a fine capillary reaching exactly to the bottom; the side tube should be situated

in the upper part of the stem. A 300 cc. beaker filled with paraffin oil or glycerol serves as the bath.

Reagents—

1. A mixture of equal volumes of glycerol and absolute alcohol, containing per cc. 15 mg. of powdered hydroxylamine hydrochloride dissolved by moderate heating.

2. Quinine hydrochloride, iron-free, U. S. P. or B. P. grade, is satisfactory.

3. Iron solution. To 25 mg. of ferrous ammonium sulfate are added 3 drops of water and 5 cc. of the glycerol-alcohol mixture. This should be freshly prepared every 2nd day.

4. Alloxan standard. An aqueous solution of alloxan monohydrate, containing 0.3 mg. per cc. If acidified with a drop of $N H_2SO_4$, and stored at 4° , it remains stable for at least 1 week.

*Procedure—*The sample under investigation is introduced into the distillation flask, and 1 cc. of the hydroxylamine solution is added, followed by sufficient alcohol to bring the volume to about 20 cc. Distillation is then started by gradual evaporation with a water pump. It is convenient to have the bath previously heated to $40-50^\circ$. Under these conditions the whole distillation takes about 12 minutes, at the end of which the temperature should have risen to $90-100^\circ$. It is further raised to $110-120^\circ$ within about 5 minutes, with occasional closing and opening of the capillary with the finger to facilitate expulsion of the last traces of moisture from the glycerol. Should any water remain on the neck of the flask, it is removed by gentle heating with a Bunsen flame.

The flask is then allowed to cool outside the bath, with the vacuum still on. When cool, exactly 4.5 cc. of absolute alcohol are added, followed by 0.2 to 0.3 gm. of solid quinine hydrochloride. The flask is thoroughly shaken for 1 minute, to insure complete solution, and finally 0.1 cc. of the iron solution is added. The blue solution is filtered, if necessary, and its extinction read in a photometer at $610 m\mu$. With pure alloxan it remains stable for hours, but with extracts from biological material it usually becomes turbid in 10 to 20 minutes.

Fig. 1 shows the recovery, with this method, of various amounts of alloxan as violuric acid. The extinction values lie very closely to the standard curve constructed by direct determination of violuric acid, a fact which proves that the conversion of alloxan into violuric acid is quantitative under these conditions. Since the molecular weights of alloxan monohydrate (160) and violuric acid (157) are nearly identical, for practical purposes each mg. of alloxan can be assumed to yield 1 mg. of violuric acid. In the construction of the curve of Fig. 1, however, the theoretical relationship has been taken into account.

Violuric acid can thus be used as a convenient and stable standard for

the photometer or the Duboscq type colorimeter. Nevertheless, each analyst should check the efficiency of the condensation procedure by plotting his calibration curve from an alloxan standard and comparing it with that obtained by direct determination of violuric acid, as has been done here. Each mg. of alloxan should give the same color as 0.97 gm. of violuric acid.

Blank—With pure alloxan solutions, a blank determination is not necessary, since no color is obtained from the reagents. Extracts of biological materials, prepared by the method outlined later, yield a light brown color which lends a greenish tinge to the pure blue of iron violurate. The extinction of these brown solutions, when measured with Filter 610, corresponds to that of 0.01 to 0.02 mg. of alloxan. If high accuracy is desired, or if amounts of alloxan smaller than 0.1 mg. are to be determined, this blank value must be taken into account. This is done by measuring the absorption of the filtered final solution before and after the addition of iron and using the difference between both readings for calculation from the standard curve.

Specificity of Method—All N-substituted alloxans yielded blue iron violurates with the present procedure, which can thus be used for the determination of these partly diabetogenic (8) compounds. The first reduction product of alloxan, dialuric acid, was likewise converted up to 80 to 90 per cent into violuric acid; this can be explained by the fact that dialuric acid is readily autoxidized to alloxan (9). Accordingly, any dialuric acid which might be present in biological material is also determined, but this can hardly be considered a setback, since this compound has been shown by Brückmann and Wertheimer (8) to possess the same diabetogenic action as alloxan, into which it seems to be rapidly converted *in vivo* by blood hemoglobin. Alloxantin, the condensation product of 1 molecule of alloxan and of dialuric acid, dissociates in aqueous solution into these compounds (10) and accordingly is recovered as violuric acid. Alcoholic solutions of alloxantin gave only traces of violuric acid.

No color was obtained with alloxanic acid, parabanic acid, murexide, uric acid, barbituric acid, and phenobarbitone. Ninhydrin gave a brown color, which it may be possible to use for the quantitative determination of this compound. A large number of blood and organ extracts from various sources never yielded a blue color. This proves, incidentally, the absence of appreciable amounts of alloxan in normal tissues, which is in contradiction to the recent report by Ruben and Tipson (11).

Application of Method to Biological Material

Obviously, a satisfactory extraction method should give the maximum of alloxan and a minimum of interfering substances, at the same time in-

suring removal of all proteins. Alloxan is easily soluble in water and acids, so that Folin-Wu or trichloroacetic acid extracts should be expected to give good results. These filtrates, however, were found to contain much interfering material, and all attempts to remove it were unsuccessful. Ammonium sulfate, followed by alcohol, was much better in this respect, and treatment with fullers' earth, which does not absorb alloxan, removed nearly all interfering substances. The procedure finally adopted for the extraction of alloxan from biological material is as follows:

Tissues—1 gm. of tissue, freshly excised or frozen in a CO₂-ether mixture, is immediately placed in a small, ice-cooled mortar, rapidly minced, and ground with glass powder with the addition of 0.5 gm. of ammonium sulfate. The mixture is then extracted by successive portions of ice-cold absolute alcohol, a total volume of 20 cc. being used. The extracts, together with the residue, are transferred to a centrifuge tube, vigorously shaken for 2 minutes, and centrifuged for 5 minutes. The supernatant is decanted into a second tube containing 1 gm. of fullers' earth (free from alkali) and 2 drops of *N* HCl, and the tube is shaken for 1 minute and again centrifuged. The filtrate is now ready for analysis, but may be kept for 24 hours in the refrigerator without loss of alloxan.

Blood or Serum—Freshly drawn blood is immediately placed in an ice-cooled test-tube containing an anticoagulant; heparin, oxalate, and fluoride are all equally satisfactory. 1 cc. is quickly pipetted into a large centrifuge tube, 0.5 gm. of solid ammonium sulfate and 20 cc. of cold absolute alcohol are added, and the mixture is shaken for 3 minutes. After centrifugation, the sample is ready for analysis and needs no treatment with fullers' earth, since blood and serum filtrates do not contain interfering substances.

Table I shows the results of some typical experiments in which 0.3 mg. of alloxan was added to various extracts prepared as outlined above. The over-all range of recovery, as determined on some 50 samples of blood, serum, plasma, and various organs is from 70 to 95 per cent. For practical purposes it is therefore suggested that alloxan values found in analyses of similar biological material be multiplied by 1.25 to correct for incomplete recovery. This correction, which is necessitated by the loss of alloxan caused by interfering substances in the alcohol extracts, may be termed the "filtrate correction."

A second, minor source of alloxan loss was discovered when alloxan, dissolved in 0.75 cc. of water, was added to 20 cc. of absolute alcohol, centrifuged, and the alloxan content of the filtrate determined. Rather unexpectedly a constant loss of 10 per cent was encountered, regardless of the amount of alloxan added. With 50 per cent alcohol the loss was still greater, but addition of 40 instead of 20 cc. of absolute alcohol resulted in

100 per cent recovery. Since, however, it seems impracticable to use more than 20 cc. of alcohol per gm. of tissue, no alteration in the procedure is proposed.

No correction has been made throughout this paper for this loss, which is probably due to the insolubility of alloxan hydrate in alcohol, the compound actually dissolved being alloxan anhydride (Biltz (12)). Experiments with a few N-substituted alloxans indicate a higher degree of insolubility in alcohol, so that with such substances appropriate corrections should be employed.

TABLE I
Recovery of Alloxan (0.3 Mg.) Added to Alcoholic Tissue Extracts

Source of extract, 1 gm. or 1 cc.	Age of sample	Alloxan recovered	Recovery
		γ	<i>per cent</i>
Serum, cat.....	Fresh	230	77
" man.....	"	275	91
" rabbit.....	1 day	260	88
Blood, "	Fresh	218	73
" "	1 day	255	86
" rat.....	Fresh	245	82
" man.....	"	275	91
Pancreas, cat.....	"	222	74
" rat.....	"	214	71
Liver, rat.....	"	214	71
Kidney, rat.....	"	268	89
Muscle, cat.....	"	275	91

Recovery of Alloxan from Biological Materials

Alloxan disappears very rapidly from biological materials. This has already been observed by previous workers (1-3) who stressed the necessity of speed in the extraction of alloxan. Knowledge of the factors responsible for this disappearance is essential for an understanding of the fate of alloxan in the body, and may thus be of value for the elucidation of the specific diabetogenic action of this compound.

While a more detailed report on the interaction of alloxan and tissues and its distribution in the body after injection will be given in a later publication, the object of the present paper is to show to what extent recovery of alloxan may be expected when it is added to minced tissues, blood, or serum.

If not indicated otherwise, all samples were used immediately after being taken, or were kept frozen in a CO₂-ether mixture for up to 30 minutes. When acidification was desired prior to the addition of alloxan,

KH_2PO_4 (pH 4.5) or HCl was added. After short periods of contact, either in test-tubes with gentle shaking or in mortars with stirring, alloxan was extracted by the ammonium sulfate-alcohol-fullers' earth process.

Table II lists a number of such experiments carried out at about 4° , since it was found that usually somewhat better recoveries were obtained at a lower temperature, which likewise slowed the rate of disappearance of

TABLE II

Recovery of 0.3 Mg. of Alloxan Added to Biological Material

Amount of tissue, 1 gm. or 1 cc.; time of contact, 5 to 10 seconds; temperature, 4° .

Sample No.	Tissue	Species	Age of sample	pH	Alloxan recovered*	Recovery
					γ	per cent
1	Serum	Man	Fresh	Natural	76	25
1	"	"	"	1-2	90	30
1	"	"	2 days	Natural	170	57
2	"	Cat	Fresh	"	161	53
3	"	Rabbit	1 day	"	190	63
4	Blood	"	Fresh	"	130	43
4	"	"	1 day	"	121	40
5	"	Man	Fresh	"	103	34
5	"	"	"	4.5	121	40
6	"	"	1 day	Natural	142	48
6	"	"	1 "	4.5	148	49
7	Pancreas	Rat	Fresh	Natural	138	46
8	"	Cat	"	"	106	35
9	Liver	Rat	"	"	115	38
10	"	Rabbit	"	"	127	42
10	"	"	"	4.5	117	39
11	Kidney	Rat	"	Natural	65	22
12	Muscle	Cat	"	"	76	25
12	"	"	"	1-2	60	20

* "Filtrate correction" (see the text) applied.

alloxan from the mixtures. In these experiments, the actual "filtrate correction" (see above) was determined for each sample by direct analysis.

While fairly large differences exist in the alloxan-binding power of the various samples, it is evident that even at low temperature and very short contact times more than half of the alloxan added disappears immediately. Somewhat better results were obtained when 5 mg. instead of 0.3 mg. of alloxan were added per gm. of tissue, but such concentrations are not encountered in actual injection experiments. Acidification prior to the addition of alloxan had usually little influence on recovery; however, keeping the samples for 1 to 2 days at 4° before use resulted frequently in a decrease in their alloxan-binding power.

Archibald (3), in his extensive review of the known properties and reactions of alloxan, comes to the conclusion that at least two factors may account for the disappearance of alloxan from biological material: (1) alkalization, with formation of alloxanic acid, (2) reduction to alloxantin by the SH— and α -amino groups of proteins or free amino acids. The same two mechanisms had already been suggested by Labes and Freisburger (13). The influence of the first factor can be avoided by the use of acidified tissues, as shown above. Thus the large and immediate loss which occurs nevertheless with fresh tissues cannot be due to alkalization. It remains to be seen whether it is caused by the immediate reduction, by SH— or other groups, of alloxan to dialuric acid, with subsequent formation of alloxantin and murexide, or whether some other reaction is involved.

SUMMARY

1. The color reaction between violuric acid and ferrous salts has been studied, and a photometric method for the determination of violuric acid has been developed.

2. Alloxan is quantitatively converted into violuric acid by evaporation *in vacuo* in the presence of hydroxylamine. It can thus be determined as violuric acid.

3. The method is specific for alloxan, N-substituted alloxans, and dialuric acid, and amounts of alloxan ranging from 0.04 to 0.4 mg. can be determined.

4. Treatment of blood, serum, or tissues with ammonium sulfate, alcohol, and fullers' earth yields extracts from which added amounts of alloxan are satisfactorily recovered. Several other extraction methods have been found less suitable.

5. Recovery experiments show that a large percentage of alloxan disappears immediately after its addition to biological material, even at low temperature and acid reaction.

I wish to express my thanks to Mr. M. Chaimowitz for his valuable technical assistance during the development of this method.

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THE METABOLISM OF SULFUR

XXXI. THE DISTRIBUTION OF URINARY SULFUR AND THE EXCRETION OF KETO ACIDS AFTER THE ORAL ADMINISTRATION OF SOME DERIVATIVES OF CYSTINE AND METHIONINE TO THE RABBIT*

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The enzymatic systems which effect the biological degradation of the sulfur-containing amino acids of the protein molecule, cystine and methionine, are not well understood. Knowledge of the intermediary stages in the metabolism of these amino acids is also lacking. When either of these amino acids is administered orally or parenterally in moderate amounts to the more common laboratory animals (cat, dog, rabbit, or rat), the sulfur is excreted almost exclusively as oxidized (sulfate) sulfur in the urine, and slight increases only in the organic sulfur fraction of the urine are observed.

It is generally believed that a primary change in the metabolism of the naturally occurring amino acids is oxidative deamination; enzyme systems which accomplish this oxidation have been described (1), and the products of such deamination have been isolated, not only in *in vitro* experiments with tissue slices (2) but also from the urine of experimental animals to which amino acids were administered (3). The keto acid derived from the oxidative deamination of methionine has been thus characterized but, so far as is known to us, the corresponding derivative of cystine has not been identified as a product of the intermediary metabolism of this amino acid.

A second problem of catabolism must be considered also in the study of the sulfur-containing amino acids; i.e., the oxidation of the sulfur. Does oxidation of the sulfur occur prior to oxidative deamination? What is the relation, if any, between oxidative deamination and oxidation of the sulfur? One of us (L.) has shown that if the deamination of the amino group of cystine (4, 5) or methionine (6) is interfered with by "blocking" the amino group with a group difficult to remove biologically (phenylureido or benzoyl), the oxidation of the sulfur does not occur normally. When these compounds were administered to rabbits, no significant increase in the oxidized (sulfate) sulfur of the urine was observed and the sulfur of the compounds administered appeared largely in the organic sulfur fraction of the urine. This relative stability of the sulfur in derivatives of cystine or

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cysteine in which the thiol group, the amino group, or both groups are blocked, has also been demonstrated by Sherwin and his coworkers (7).

The development of methods for the determination of α -keto acids in biological material (3, 8) has made possible a new approach to the problem of the metabolism of the sulfur-containing amino acids. If oxidative deamination of the S-substituted derivatives of cysteine or homocysteine has occurred, and if further oxidation (*i.e.* of the sulfur) was prevented, the α -keto acid might be excreted in the urine and the sulfur of this compound would contribute to the urinary organic sulfur. Furthermore, it has been demonstrated that in the metabolism of the aromatic S-substituted derivatives of cysteine and homocysteine, the amino group may be acetylated and the acetylated product (mercapturic acid) may be excreted in the urine (9).¹ The "extra" organic sulfur of the urine in such experiments might thus include (1) the sulfur of the compound administered, (2) the sulfur of the α -keto acid, and (3) the sulfur of mercapturic acid.

The purpose of the present study was to investigate the distribution of the urinary sulfur after administration of certain derivatives of cysteine, homocysteine, and methionine not previously studied and to correlate the oxidation of the sulfur with the deamination so far as was possible. The experiments have demonstrated that deamination of such compounds as S-benzylcysteine and S-benzylhomocysteine occurs without oxidation of the sulfur to sulfate sulfur and that the α -keto acids thus formed are excreted in the urine.

EXPERIMENTAL

The general procedures and analytical methods were those used in previous studies of the oxidation of sulfur compounds (6). Adult male rabbits, 2 to 3 kilos in weight, were kept in metabolism cages and were fed 75 gm. of cabbage and 45 gm. of oats daily. The daily food was consumed completely except in a very few instances on the days when the compounds were fed.

The compounds, for the most part difficultly soluble in water, were dissolved in a small volume of water with the aid of sodium hydroxide, the amount of alkali used being slightly in excess of the theoretical, and these solutions were administered through a stomach tube. With S-benzylcysteine and S-benzylhomocysteine, it was not possible to effect complete solution in this way readily and the compounds were fed partly as the sodium salt and partly as the amino acid derivative in suspension.

Keto acids were estimated by Shacter's modification² of the method of Case (8). The day's specimen of urine was diluted to 250 to 300 ml. and 10 ml. of this were further diluted to 50 ml. 5 to 10 ml. of this last diluted

¹ Witter, R. F., unpublished data.

² Shacter, B., unpublished data.

urine were placed in a separatory funnel (125 ml. capacity) and 1 ml. of the 2,4-dinitrophenylhydrazine reagent³ was added. After 15 minutes, the solution was extracted with 25 ml. of redistilled benzene (3 minutes shaking).

The aqueous layer was drawn off and the benzene extract was washed with 5 ml. of water, which was drawn off and discarded. 20 ml. portions of the benzene extract were transferred to a clean separatory funnel and were extracted three times with 10, 5, and 5 ml. portions of a borate buffer⁴ (pH 8.8). Small amounts (2 ml.) of the buffer were added after each extraction and were drawn off immediately without shaking in order to wash out the stem of the funnel. The extract and washings were combined in a 25 ml. glass-stoppered graduate, diluted to 25 ml. with borate buffer, and after thorough mixing, 10 ml. aliquots were pipetted into 25 ml. volumetric flasks. After the addition of 10 ml. of 2 N sodium hydroxide, the contents were diluted to 25 ml. with water and thoroughly mixed. After 90 seconds, the intensity of the color developed was measured by means of an Evelyn photoelectric colorimeter with the use of filter No. 520. The amount of keto acid was calculated from the standard value of *K* obtained by applying the same procedure to aqueous solutions of sodium phenylpyruvate⁵ of known concentration.

It was not possible to secure the keto acid derivatives of all the compounds studied for use as standards. It was accordingly decided to express all keto acid values in terms of phenylpyruvic acid. The amount of sulfur excreted as the keto acid of the derivatives fed was then calculated on the assumption that all the extra keto acid excreted was derived from the compound fed.

A series of other α -keto acids⁶ (pyruvic (sodium salt), *o*-nitrophenylpyruvic, α -keto- β -methylvaleric, α -ketoisocaproic, and α -keto- γ -methylthiolbutyric acids) was tested by the procedure outlined. The intensities of the colors developed by equimolar solutions of phenylpyruvic acid and the other α -keto acids agreed within 10 per cent. This is in confirmation of the results of Waelsch and Miller (3).

The *dl*-methionine, *dl*-*N*-methylmethionine, and the *dl*-*N*-benzoylmethio-

³ 0.2 gm. of 2,4-dinitrophenylhydrazine was dissolved in 70 to 80 ml. of 2 N hydrochloric acid with warming and the solution was diluted with water to 100 ml.

⁴ A mixture of 8 gm. of borax and 3 ml. of concentrated hydrochloric acid was dissolved in water to make a volume of 1 liter.

⁵ An amount of the monohydrate of sodium phenyl pyruvate (0.0622 gm.), which was equivalent to 50 mg. of the acid, was dissolved in water and the solution was diluted to a liter. 10 ml. of this solution were equivalent to 0.5 mg. of phenylpyruvic acid.

⁶ We are indebted to Dr. R. W. Jackson for the sample of *o*-nitrophenylpyruvic acid, to Dr. W. C. Rose for the α -keto- β -methylvaleric and α -ketoisocaproic acids, and to Dr. W. M. Cahill for the α -keto- γ -methylthiolbutyric acid.

nine were prepared in this laboratory by Dr. J. M. Burkhalter; the *dl*-S-benzylcysteine, *dl*-S-benzylhomocysteine, and S-carboxymethylcysteine were similarly prepared by Dr. F. R. Blood, and S-phenylcysteine by Dr. R. F. Witter. Inactive⁷ homocystine was obtained from Dr. S. A. Lough of the University of Nevada and ethionine from Dr. Vincent du Vigneaud of Cornell University Medical College. The A. E. Staley Manufacturing Company supplied the *l*-methionine. That these compounds were of satisfactory purity was demonstrated by analyses for sulfur and nitrogen.

The results of typical experiments are summarized in Table I. It will be noted that in the experiments with *l*-methionine the greater part of the extra sulfur of the urine was present in the oxidized (sulfate) form and that the excretion of extra keto acid was negligible. The ease of oxidation of the sulfur is in agreement with the results previously obtained in this laboratory with rabbits fed *dl*-methionine (6). It is to be noted also that, in two other experiments with the animals (Nos. 1 and 3) in which amounts of *dl*-methionine comparable to those of Table I were fed, the extra keto acid excretions were 4 and 8 mg. respectively as compared to 2 and 8 mg. (Table I) when *l*-methionine was fed. This ready oxidation of *dl*-methionine as compared with that of the *l* isomer was not unexpected since the isomeric *d*- and *l*-methionine are reported to be equally well utilized for growth by the white rat (11, 12).

The sulfur of *dl*-N-monomethylmethionine appeared also to be readily and completely oxidized, the distribution of the extra urinary sulfur being similar to that observed previously after the administration of *dl*-methionine (6) and in the present series with *l*-methionine (Table I). No significant increases in the excretion of keto acids were observed. Similar results were obtained in two other experiments not detailed in Table I. It is usually assumed that the N-monomethyl amino acids are oxidatively deaminized and behave similarly in metabolism to the unsubstituted amino acids. *dl*-N-Monomethylmethionine (13) and N,N'-dimethyl-*l*-cystine (14) are both effective in the promotion of growth of rats on a diet deficient in sulfur-containing amino acids. In view of these observations, the ready oxidation of the sulfur of N-monomethylmethionine was not unexpected.

It has previously been demonstrated in this laboratory (6) that when the α -amino group of methionine is blocked by a benzoyl group, a group not readily removed in metabolic activity, the oxidation of the sulfur does not occur readily. In two experiments, not presented in Table I, in which N-benzoylmethionine was fed, the changes in the keto acid excretion were so slight as to be within the error of the experiment. Similarly, in an experiment with S-carboxymethylcysteine, whose sulfur is not oxidized

⁷ Homocystine was prepared by the demethylation of *dl*-methionine. It is not known whether this is the *dl* isomer or the meso isomer or a mixture of the two (10). For convenience, the compound will be referred to as inactive homocystine.

readily when the compound is administered to the rabbit (15), no increases in the keto acid excretion were noted.

TABLE I

Excretion of Extra Sulfur and Extra Keto Acids in Urine after Oral Administration of Derivatives of Cystine and Methionine

With the exception of ethionine, the amount of each compound fed was the equivalent of 430 mg. of sulfur (with ethionine, 143 mg. of S). In the first column the first value is the number of the experimental animal and the second the number of the experiment. In the column headed total sulfur, the values in parentheses represent the extra total S excretion expressed as percentage of the amount fed and the values in parentheses in the sulfate and organic sulfur columns the percentage distribution of the extra total sulfur as extra sulfate and extra organic sulfur. Keto acids are calculated as phenylpyruvic acid and as sulfur on the assumption that all the extra keto acid is derived from the sulfur-containing compound fed. In all the experiments, the first horizontal line represents excretions of the first 24 hour period and the second those of the entire period following administration of the compounds during which extra sulfur was excreted.

Animal and experiment Nos.	Compound fed	Extra S			Extra keto acid		
		Total	Total SO ₄	Organic (a)	Keto acid	As S equivalent (b)	(b)/(a) × 100
		mg.	mg.	mg.	mg.	mg.	
1-4	l-Methionine	134 (31)	102 (76)	32 (24)	2	0.4	1
3-5	"	302 (47)	159 (79)	43 (21)	8	1.5	3.5
		357 (90)	315 (81)	72 (19)	12	2.3	3
1-5	dl-N-Methyl-methionine	105 (24)	79 (75)	26 (25)	1	0.2	<1
		267 (62)	212 (79)	55 (21)	1	0.2	<1
3-6	" "	187 (43)	117 (63)	70 (37)	4	0.7	1
		402 (93)	284 (71)	118 (29)	7	1.3	1
3-7	Homocystine	175 (41)	93 (53)	82 (47)	28	5.5	7
		211 (49)	87 (41)	124 (59)	43	8.5	7
5-1	"	162 (38)	86 (53)	76 (47)	27	5.4	7
		260 (60)	125 (48)	135 (52)	44	8.6	6
3-8	dl-S-Benzyl-homocysteine	91 (21)	-26 (-29)	117 (129)	168	32.8	28
		371 (86)	54 (15)	317 (85)	298	58.1	18
4-1	" "	232 (54)	33 (14)	199 (86)	219	42.8	21
		278 (65)	37 (13)	241 (87)	221	43.2	18
6-2	Ethionine	102 (72)	7 (7)	95 (93)	38	7.4	8
		165 (115)	-6 (-4)	171 (104)	65	12.6	7
6-3	dl-S-Benzyl-cysteine	198 (46)	36 (18)	162 (82)	92	18.0	11
		332 (77)	107 (32)	225 (68)	105	20.4	9
10-1	" "	142 (33)	-1 (-1)	143 (101)	76	14.8	10
		189 (44)	19 (10)	170 (90)	76	14.8	9

Our results indicate a less ready oxidization of the sulfur of inactive homocystine as evidenced by the excretion of extra urinary sulfate sulfur than has been reported previously by du Vigneaud and coworkers (10).

This may be explained by the larger amount of homocystine fed (1.8 gm. as compared with 0.5 gm.). A definite increase in keto acid was observed, which corresponded to approximately 7 per cent of the extra organic sulfur. It may be noted that in experiments with tissue slices (liver, kidney) of rats (16), no evidence of the deamination of either homocystine or homocysteine as shown by the formation of keto acid could be demonstrated. Our experiments suggest that inactive homocystine is less readily metabolized by the rabbit than are *dl*-methionine and *l*-cystine.

Ethionine, the homologue of methionine which contains an S-ethyl group rather than an S-methyl group, will not promote the growth of rats on a cystine-deficient diet, a striking example of the specificity in the biological reactions of the S-containing amino acids (17). Further evidence of the biological inertness of ethionine is afforded by the single but striking experiment recorded in Table I. 72 per cent of the sulfur of the orally administered ethionine was excreted within 24 hours after its administration but of this almost the entire amount (93 per cent) appeared as organic sulfur. The keto acid excretion was increased, corresponding to about 8 per cent of the extra organic sulfur of the urine. It appears that ethionine is not readily metabolized, even though some deamination has occurred. The behavior of ethionine is in striking contrast to that of another type of analogue of methionine, S-methylcysteine. Although the latter cannot support the growth of rats in lieu of cystine (10, 18), which is presumed to indicate a failure of demethylation, its sulfur is excreted in large part as sulfate sulfur after oral administration to rabbits (10). Studies of the action of the amino acid oxidases on S-ethylcysteine, a homologue of ethionine, have shown that both the *l* isomer (1) and the *dl* isomer (2) are rapidly oxidized.

In addition to ethionine, two other S-substituted derivatives of amino acids were studied in some detail, S-benzylhomocysteine and S-benzylcysteine. With these compounds in which a non-labile group, the benzyl group, is attached to the sulfur, no significant oxidation of the sulfur was observed. There were no increases in the oxidized sulfur of the urine beyond the error of the experimental procedure, and the entire increase in urinary sulfur was in the organic sulfur fraction. Despite this failure of oxidation of the sulfur, considerable deamination occurred, since the excretion of keto acids corresponded to 28 and 21 per cent of the extra organic sulfur with S-benzylhomocysteine and 11 and 10 per cent with S-benzylcysteine. The failure of oxidation of the sulfur of S-benzylcysteine is in confirmation of experiments in the literature (7, 19) and unpublished results from this laboratory.¹ We have also studied the distribution of urinary sulfur¹ and the keto acid excretion after feeding 0.75 gm. of S-phenylcysteine to two rabbits. The results were similar to those discussed above, *i.e.* a sharp increase in keto acid excretion and failure of oxida-

tion of the sulfur, all of the extra sulfur being present in the organic sulfur fraction of the urine.

It has been demonstrated that the S-benzyl derivatives of cysteine (9, 20)¹ and homocysteine (9, 20) are acetylated and are excreted in the urine as mercapturic acids. Quantitative studies are lacking; the amounts of mercapturic acid isolated, while undoubtedly minimal, represented a relatively small proportion of the compounds administered. The extra organic sulfur of the urine in our experiments with these derivatives of cysteine and homocysteine is undoubtedly derived from at least three sources, the unchanged derivative, the acetylated compound (mercapturic acid), and the deaminized amino acid (α -keto acid). We have quantitative data on the last of these only.

Since the analytical data indicated a very considerable excretion of α -keto acids after the administration of *dl*-S-benzylcysteine and *dl*-S-benzylhomocysteine, attempts were made to isolate the 2,4-dinitrophenylhydrazine derivatives of the keto acids from the urine by a procedure similar to that used by Waelsch and Miller (3).

Although an initial precipitation of the hydrazone was readily obtained, it was difficult to secure a clean crystalline material when the crude derivatives were recrystallized. Success was finally achieved by adsorption on a column of alumina (prepared according to Brockmann, Merck and Company). The chromatogram was developed by repeated washings with a mixture of ethyl acetate and ethanol (2:1 followed by 1:1). After the final washing, a small brownish yellow band and a yellow band were obtained. These were separated mechanically and both bands were eluted with 2 per cent sodium carbonate with gentle heating. After filtration the eluate was acidified with dilute hydrochloric acid (1:4) and the flocculent precipitate was filtered off. The precipitate was dissolved in ethyl acetate. As the solvent evaporated at room temperature, small amounts of yellow needle crystals were obtained.

The hydrazone of the α -keto- γ -S-benzylbutyric acid melted at 160–161° (corrected) and gave the following results on analysis.

	Calculated for $C_{17}H_{19}N_3O_4S$ per cent	Found per cent
Carbon.....	50.50	50.73, 50.78
Hydrogen.....	3.96	4.29, 4.26
Sulfur.....	7.92	8.04, 7.92
Nitrogen.....	13.86	12.48, 12.40

With the exception of the values for nitrogen, these results compare closely with the expected values. The low values for nitrogen may possibly be related to analytical difficulties associated with the presence of nitro groups.

A similar hydrazone obtained from the urines of rabbits fed S-benzylcysteine melted at 145.5–147° (corrected), but no microanalyses were made.

It has been shown that *l*-amino acid oxidase (1) readily attacks S-benzylcysteine but that S-carboxymethylcysteine is oxidized very slowly. The N-monomethyl derivative of methionine is also oxidized rapidly. These observations with *in vitro* experiments are supported by the present *in vivo* studies with rabbits. Borek and Waelsch (16), however, in experiments with tissue slices were unable to deaminate homocysteine or homocystine. This is difficult to explain, particularly since the N-monomethyl derivative of homocysteine has been shown to be readily oxidized by purified *l*-amino acid oxidase. It has also been possible, in the present work, to demonstrate a significant increase in the urinary keto acid excretion after feeding homocystine.

SUMMARY

The distribution of extra urinary sulfur and the excretion of α -keto acids after the oral administration of methionine and various derivatives and homologues of cysteine and methionine to rabbits have been determined.

When *dl*- and *l*-methionine and *dl*-N-monomethylmethionine were fed, most of the extra sulfur of the urine appeared in the oxidized (sulfate) sulfur fraction and there was no appreciable increase in the excretion of α -keto acids. This finding is in agreement with the usually accepted hypothesis that N-monomethyl derivatives of amino acids undergo oxidative deamination and are readily metabolized.

When S-benzyl derivatives of homocysteine and cysteine were fed, the extra urinary sulfur appeared chiefly in the organic sulfur fraction and there was no evidence of oxidation of the sulfur. That oxidative deamination occurred nevertheless was shown by the significant increase of α -keto acids in the urine and by the isolation from the urine of the 2,4-dinitrophenylhydrazones of the corresponding α -keto acids. The sulfur of inactive homocystine was less readily oxidized than the sulfur of either *dl*- or *l*-methionine and significant increases in the urinary excretion of α -keto acids were observed after the administration.

When ethionine was fed, all the extra sulfur appeared in the organic sulfur fraction of the urine, while there was also a significant rise in the urinary α -keto acids.

These results indicate that oxidative deamination of the sulfur-containing amino acids or their derivatives may occur, even though further oxidation (*i.e.*, of the sulfur to sulfate) is blocked by the presence of a non-labile substituent group (in the present instance, ethyl or benzyl) attached to the sulfur.

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THE DEVELOPMENT OF THE CYTOCHROME OXIDASE AND SUCCINOXIDASE SYSTEMS IN THE CHICK EMBRYO

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Cytochrome oxidase and succinic dehydrogenase are closely linked, functionally in the succinoxidase system, and structurally by their attachment to the same particles ("macro molecules") of cells. Only with great difficulty has it been possible to separate either of the enzymes from these particles. Stern (1), using ultracentrifugation and electrophoresis in the Tiselius cell, was unable to fractionate the enzymes from the particles obtained from beef and pig heart muscle. The first indication that the enzymes could be separated was in the work of Hopkins, Lutwak-Mann, and Morgan (2), who obtained a fraction with succinic dehydrogenase activity but no cytochrome oxidase activity. Only recently have the enzymes been obtained in solution free of cellular particles. By controlled autolysis and ultrasonic vibration, Haas (3) separated the cytochrome oxidase component of heart muscle. By treating the mitochondrial granules of guinea pig liver with 0.01 M NaHCO_3 , Hogeboom (4) separated the succinic dehydrogenase.

In view of this intimate association in the cell, any difference in the time of appearance or in the rate of development of these two enzyme systems during embryogenesis would be of interest. The changes in the development of cytochrome oxidase in chick embryos have been studied (5, 6), but no similar studies have been reported for succinic dehydrogenase. In the present work, both cytochrome oxidase and succinoxidase activities were followed to determine whether they changed in the same manner.

EXPERIMENTAL

Eggs of red rock hybrid hens were incubated at 36–37° for lengths of time varying from 25 hours to 21 days. The embryos were removed from the yolk and dissected free from the area opaca in the early stages and from all extraembryonic membranes in the later stages. Through the 5th day of incubation, the age of each embryo was determined by microscopic examination. There was considerable variability among the individual embryos of a given batch of eggs incubated for the same length of time. Those which differed too greatly from the expected age were discarded. The remaining embryos were dropped into 1 ml. of ice-cold distilled water,

weighed, diluted to a given volume with water, and homogenized by a motor-driven glass homogenizer (7). At the 21st day, when feathers and bone made immediate homogenization in this fashion difficult, the embryos were first broken up in the Waring blender for 2 minutes. The number of embryos used for a homogenate was varied to keep the rate of oxygen uptake fairly uniform, from forty-four embryos at the earliest stage (25 hours) to three embryos in the later stages (after the 7th day).

The homogenates were assayed manometrically at 37° for both succinoxidase and cytochrome oxidase activities (8). In the succinoxidase determinations, duplicates were run of each of the following three systems: (1) 0.3 ml. of 0.5 M sodium succinate, pH 7.4; 0.5 ml. of 1.52×10^{-4} M cytochrome *c* (isolated from beef heart according to Keilin and Hartree (9) and preserved in lyophilized state); 0.3 ml. of 4×10^{-3} M CaCl_2 ; 0.3 ml. of 4×10^{-3} M AlCl_3 ; 0.8 ml. phosphate buffer (0.1 M, pH 7.4); 0.4 ml. of homogenate; and enough water to make a total volume of 3.0 ml. (2) is the same as (1), except for the addition of 0.3 ml. of 0.01 M sodium azide. (3) is the same as (1), except for the absence of cytochrome *c*. In the cytochrome oxidase determinations, the following four systems were used: (1) 0.3 ml. of 0.119 M ascorbic acid, adjusted with NaOH to pH 7.4; 1.3 ml. of 1.52×10^{-4} M cytochrome *c*; 1.0 ml. of 0.1 M phosphate buffer, pH 7.4; 0.1 ml. of homogenate and enough distilled water to make a total volume of 3.2 ml. (in duplicate). (2) is the same as (1), except for the absence of cytochrome *c* (in duplicate). (3) is the same as (1), except for the addition of 0.3 ml. of 0.01 M sodium azide. (4) is the same as (1), except for the absence of both cytochrome *c* and homogenate.

Fig. 1 shows the activities of succinoxidase and cytochrome oxidase, in terms of oxygen consumption per embryo per hour, plotted against the age of the embryo. The portion of the curve for the first 6 days of development is enlarged in Fig. 2. In all graphs the age through the 4th day represents the average age of the embryos as determined morphologically. Under our conditions, this age was in most cases 23 hours less than the incubation time. All later ages indicated on the graphs were obtained by subtracting 1 day from the incubation time. (In only two batches of eggs were any incubated long enough to permit hatching. In both cases hatching occurred on the 22nd day of incubation.) In estimating cytochrome oxidase activity, the oxygen uptakes have been corrected for ascorbate autoxidation by subtracting the values obtained in the absence of cytochrome *c*.

Even the youngest embryos tested gave an appreciable oxygen uptake, almost 25 c.mm. per embryo per hour, for cytochrome oxidase. Albaum and Worley (5), using fewer embryos, with a different substrate and at a lower temperature, were unable to show any enzyme activity until the 4th day of incubation. Moog (6), using indophenol blue production in em-

bryos treated with nadi reagent, reported a positive reaction in embryos from 16 through 96 hours. . On the basis of the time elapsing before the appearance of a "standard colorization," Moog concluded, "The enzyme appears to increase in quantity up to the middle of the second day of incubation, when it reaches a level that is maintained through the fourth day." Our data indicate an increase of some 900 per cent between the 2nd and 4th days.

The values obtained for cytochrome oxidase activity in the young em-

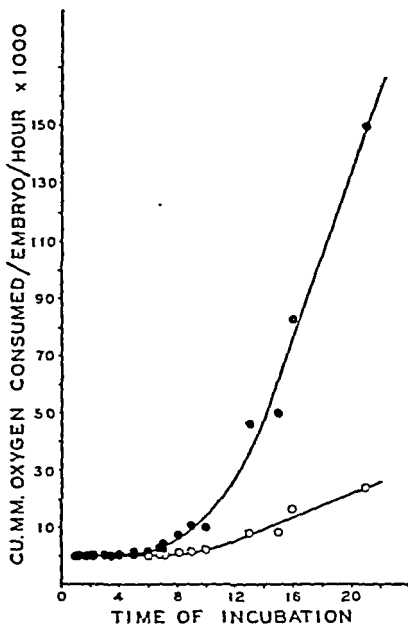


FIG. 1. The development of the cytochrome oxidase (●) and the succinoxidase (○) systems in the chick embryo between 1 and 21 days of incubation.

bryos (1 to 3 days) may be compared with data for the respiration of intact isolated embryos reported by Philips (10). In all cases the activity, in terms of c.mm. of O₂ per embryo per hour, is high enough to account for the total respiration of the intact embryo. Although we obtained oxygen uptakes for the succinoxidase system in all but the youngest embryos (25 hours), the values were low and in no case sufficient to account for the total respiration of the embryo.

To demonstrate the relationship of cytochrome oxidase and succinoxidase

activities during development, the ratio of the oxygen uptakes through the two systems was calculated. Fig. 3 shows this ratio plotted against the age of the embryo. The ratio is high to begin with and then drops to a level which remains fairly constant from about the 5th to the 21st day.

Thus, in the chick embryo, both the time of appearance and rate of development of cytochrome oxidase activity and succinoxidase activity are different. Only after the 5th day does the ratio of these activities become constant. The low succinoxidase activity in the early embryos is to be

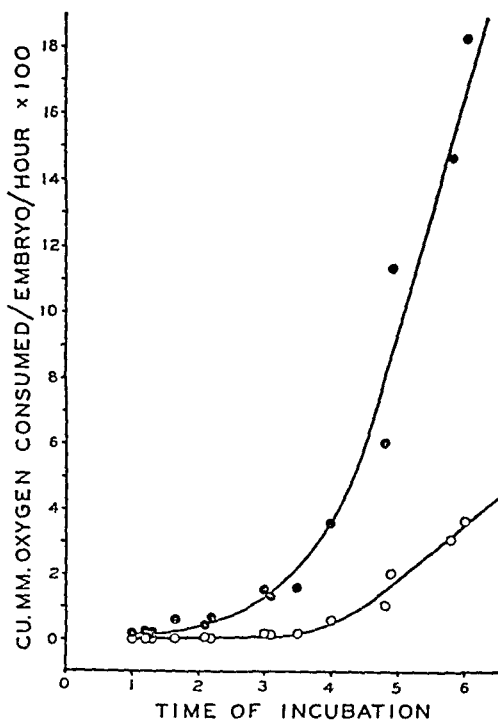


FIG. 2. The development of the cytochrome oxidase (●) and the succinoxidase (○) systems in the chick embryo between 1 and 6 days of incubation.

explained by a low limiting concentration either of succinic dehydrogenase or another system which links the succinic dehydrogenase to the cytochrome oxidase system. (Stern (1) suggested that a protein which he separated from the cellular particles of heart muscle acted as a "coupling link between succinic dehydrogenase and the cytochrome-cytochrome oxidase system." Ultracentrifugally purified particles were unable to catalyze the aerobic oxidation of succinate, though able to do so for either hydroquinone or *p*-phenylenediamine; in the presence of an H acceptor like methylene blue they did, however, catalyze the oxidation of succinate to fumarate.)

It appears that, before the 5th day, the chick embryo has not developed completely the enzyme complex attached to the cellular particles. Similar independence in the development of cytochrome oxidase and succinoxidase has been found in rat liver by Potter.¹ The ratio of cytochrome oxidase to succinoxidase 3 days before birth is 3.7, falls to about 1.5 near birth, and rises to about 6.0 some 8 days after birth.

Azide Inhibition—The inhibition of cytochrome oxidase and succinoxidase activities by 0.001 M sodium azide was found to remain fairly constant between the 3rd and 21st days of development. But, despite the fact that cytochrome oxidase, for which azide is considered a specific inhibitor, is

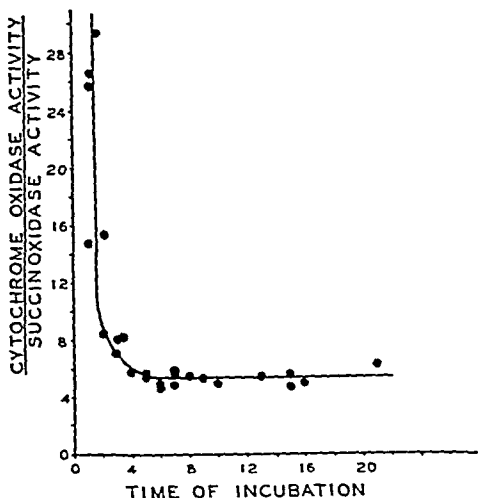


FIG. 3. The relationship in the development of cytochrome oxidase to succinoxidase activity in the chick embryo between 1 and 21 days of incubation.

operating in both systems, there is a striking difference in level of inhibition of the two systems, 68 ± 3.8 per cent² inhibition for cytochrome oxidase and 25 ± 6.1 per cent² inhibition for succinoxidase.

Commoner (11) observed that the per cent inhibition of yeast respiration by cyanide was smaller at lower substrate concentrations. If we consider that the reduced cytochrome *c* is the substrate for cytochrome oxidase, we may have a comparable situation here. In the cytochrome oxidase system, the excess ascorbate maintains the level of reduced cytochrome *c*, so that the per cent inhibition is high. But in the succinoxidase system, if the

¹ Potter, V. R., private communication.

² S.D. = $\sqrt{\Sigma d^2/n}$.

succinic dehydrogenase is the limiting factor and is present in relatively low concentrations, the quantity of reduced cytochrome *c* available to the cytochrome oxidase would be lower than in the cytochrome oxidase system. There consequently would be a reduced per cent inhibition.

The azide inhibition of cytochrome oxidase activity in embryos less than 3 days old was found to be lower. In these young embryos the "autooxidation" of ascorbate represents a large fraction of the observed oxygen uptake. The precision of our manometric technique and the questionable validity of ascorbate "autooxidation" blanks in the presence and absence of azide do not warrant our considering these early low results to represent a trend.

SUMMARY

1. The cytochrome oxidase and succinoxidase activities of chick embryos, ranging in age from 25 hours to 21 days, were measured manometrically.

2. Both the time of appearance and the rate of development of cytochrome oxidase activity and succinoxidase activity are different. The ratio of cytochrome oxidase activity to succinoxidase activity is high to begin with and then drops to a level which remains fairly constant from the 5th to the 21st day of development.

3. The azide inhibition of the two systems is fairly constant between the 3rd and 21st days of development. The difference in level of inhibition (68 ± 3.8 per cent for the cytochrome oxidase and 25 ± 6.1 per cent for the succinoxidase) is interpreted in terms of a substrate effect.

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THE OXIDATION OF HIGHER FATTY ACIDS IN HEART MUSCLE SUSPENSIONS*

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It is now well known that the liver is capable of oxidizing higher fatty acids with the formation of the ketone bodies. Since the liver has only a limited ability to metabolize the ketones, these substances diffuse into the blood stream and are carried to the peripheral tissues, which are capable of the complete oxidation of the ketones to carbon dioxide and water. This two-stage mechanism of fatty acid oxidation may account for the metabolism of a considerable portion of the fatty acids undergoing oxidative degradation in the organism. Stadie, however, has emphasized in his recent review (1) that this two-stage mechanism cannot account quantitatively for the total fat undergoing oxidation in the fasting or diabetic animal. This fact has been well established by a number of investigators (quoted in Stadie's review) and it implies that the liver is not the sole site of the direct oxidation of fatty acids. It would appear, then, that extrahepatic tissues are capable of the direct oxidation of fatty acids as well as the oxidation of the ketone bodies. However, this conclusion has not gained wide-spread acceptance because of a long standing controversy over the question of the direct oxidation of fatty acids by muscle (*cf.* Gemmill (2)).

Recently the author described the experimental conditions necessary to demonstrate the oxidation of higher fatty acids by liver homogenates and washed suspensions of liver (3, 4). Fatty acid oxidation requires the presence of adenosine triphosphate (ATP), which may be added to the system as such or which may be generated *in situ* by oxidative phosphorylation of adenylic acid during the simultaneous oxidation of fumarate. Fatty acid oxidation in the liver preparations may also be coupled specifically to the oxidation of α -ketoglutarate to succinate (4). More recently the author has shown that fatty acid oxidation in washed liver preparations results in the quantitative formation of acetoacetate (*i.e.* for each mole of octanoate undergoing oxidation 2 moles of acetoacetate are formed). It was also found that fragments formed from the fatty acid during oxidation can combine with oxalacetate to form citrate, thus implicating the Krebs tricarboxylic acid cycle in the over-all process of fatty acid oxidation (5, 6). Although the details of these reaction mechanisms are not yet

* This investigation was supported by a grant from the Albert and Mary Lasker Foundation, Inc., and the Sidney and Frances Brody Foundation.

known in terms of specific enzymes and intermediates, the information at hand concerning the conditions of enzymatic fatty acid oxidation in liver preparations has presented the opportunity of surveying various extra-hepatic tissues for their ability to oxidize higher fatty acids.

This report describes the results of a study of the oxidation of higher fatty acids by rat heart muscle suspensions. These preparations are capable of the oxidation of fatty acids under conditions resembling those required to demonstrate this process in liver suspensions. Acetoacetate does not accumulate during fatty acid oxidation in heart muscle suspensions. However, acetoacetate is vigorously oxidized by these preparations. Both fatty acid and acetoacetate oxidation result in the accumulation of succinate when succinic dehydrogenase is inhibited by malonate. The accumulation of succinate is of such a magnitude that it suggests that the Krebs cycle is the major pathway of oxidation of fragments derived from both fatty acids and acetoacetate.

Rat heart muscle therefore possesses the enzymatic equipment for the direct oxidation of fatty acids.

EXPERIMENTAL

The preparation or source of the various substrates or intermediates used has been listed in previous publications of the author (3, 4, 6).

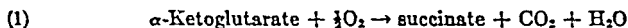
The rat heart suspensions were prepared as follows: Hearts were quickly removed from adult rats sacrificed by decapitation, chilled on cracked ice, and quickly minced with scissors. The tissue was then homogenized (7) in ice-cold buffer, the composition of which has been described (4). For each heart, 3 ml. of buffer were used. The homogenizer pestle and tube were chilled in cracked ice before use. The homogenization was carried on for about 2 to 3 minutes in a rather loose fitting tube at high speed. The homogenate was filtered into a chilled test-tube through two layers of gauze, a considerable residue of stroma being left behind. The homogenate was immediately pipetted into the previously prepared Warburg vessels.

All components of the medium were present in the main compartment of the Warburg vessel before addition of homogenate. The vessels were equilibrated at low bath temperatures (25–28°) for 5 minutes and the taps were then closed. Oxygen consumption data were extrapolated back to the time of addition of the homogenate, allowing one-half maximum activity for the initial equilibration period.

Acetoacetate, citrate, and the sum of α -ketoglutarate plus succinate were determined as described in a previous paper (6). Fatty acids or acetoacetate did not give rise to more than traces of succinate as a result of acid permanganate treatment in the latter determination.

The amounts of oxygen, substrates, etc., are expressed in terms of c.mm. of ideal gas, 22.4 c.mm. being equal to 1.0 micromole.

Requirements for Demonstration of Fatty Acid Oxidation in Heart Muscle Homogenates—Fatty acid oxidation in liver preparations requires either (a) ATP; (b) the oxidative metabolism of intermediates of the Krebs tricarboxylic acid cycle in the presence of adenine nucleotide; or (c) the specific oxidative reaction



with or without added adenine nucleotide (4). Although (b) and (c) are known to cause oxidative phosphorylation of adenine nucleotides to ATP, there is an indication that the formation of ATP does not necessarily account for the activation of fatty acid oxidation by reaction (c) (4).

The ability of heart muscle homogenate to cause extra oxygen uptake in the presence of octanoate was therefore measured under the three different conditions outlined above. The reaction media resembled those found adequate for the liver system. They contained magnesium sulfate, sodium malonate, phosphate buffer, and in some cases cytochrome c.

In Table I are shown the results of these experiments. It can be seen that the addition of ATP alone failed to activate octanoate oxidation. Likewise, α -ketoglutarate plus ATP failed to yield extra oxygen uptake in the presence of fatty acid. However, when octanoate, laurate, or palmitate was added to a heart muscle suspension oxidizing fumarate in the presence of ATP a considerable extra oxygen uptake ensued. There was no oxygen uptake when the enzyme suspension was omitted from the reaction medium. Fumarate oxidation in the absence of ATP did not activate fatty acid oxidation. Myoadenylic acid could be substituted for ATP. Oxalacetate was not an effective substitute for fumarate. Therefore, fumarate oxidation in the presence of adenine nucleotide is a necessary factor in the demonstration of fatty acid oxidation in heart muscle homogenates.

When malonate was omitted from the reaction medium, ATP alone gave an extra O_2 uptake in the presence of octanoate. Since malonate inhibits the endogenous respiration of the suspension greatly (60 to 80 per cent), it is probable that the positive effect noted with ATP alone in the *absence* of malonate is actually due to fumarate oxidation, the source of fumarate being metabolites present in the heart muscle suspension itself. That this explanation is probably correct is indicated by Experiment 4 in Table III. In that experiment succinate accumulated when the endogenous respiration was inhibited by malonate, which inhibits succinic dehydrogenase. Since succinate oxidation normally leads to fumarate, it is therefore probable that the positive effect given by ATP *alone* in the absence of

malonate was due to fumarate oxidation. The use of malonate to inhibit the considerable endogenous oxygen uptake of the suspension allowed a closer study of the factors affecting fatty acid oxidation. In addition,

TABLE I

Requirements for Fatty Acid Oxidation in Heart Muscle Homogenates

The Warburg vessels contained 1.0 ml. of homogenate, 0.20 ml. of MgSO_4 (0.005 M),* 0.20 ml. of sodium malonate (0.01 M), 0.20 ml. of sodium adenosine triphosphate (0.0011 M), 0.05 ml. of phosphate buffer, pH 7.4 (0.007 M), 0.05 ml. of cytochrome c (1×10^{-5} M), 0.10 ml. of H_2O or octanoate (0.001 M) or laurate (0.001 M) or palmitate (0.0005 M) and additions, as indicated below, of 0.20 ml. of H_2O or α -ketoglutarate (0.002 M) or fumarate (0.005 M) or oxalacetate (0.005 M). When components were omitted, H_2O was substituted. Total volume 2.00 ml.; alkali in center well; temperature 25° ; time 2 hours.

Experiment No.	Substrate	Additions	O ₂ uptake
			c.mm.
1	None	None	50
	Octanoate	"	46
2	None	α -Ketoglutarate	100
	Octanoate	"	105
3	None	Fumarate	330
	Octanoate	"	522
	Laurate	"	480
	Palmitate	"	442
4	None	" (adenosine triphosphate omitted)	112
	Octanoate	Fumarate (adenosine triphosphate omitted)	96
5	None	Fumarate (myoadenylic acid substituted for adenosine triphosphate)	375
	Octanoate	"	501
6	None	Oxalacetate	211
	Octanoate	"	247
7	None	Fumarate (enzyme omitted)	-1
	Octanoate	" " "	0
8	None	None (malonate omitted)	170
	Octanoate	" " "	312

* The figures in parentheses indicate the concentration of the component in the complete reaction medium. This notation is used in all tables in this paper.

malonate served the purpose of causing succinate accumulation in those experiments in which this end was desired for analytical purposes.

When the oxygen uptakes of the fumarate-supplemented system are plotted against time (see Fig. 1), it can be seen that there is very little extra oxygen uptake in the presence of octanoate for some 20 minutes after addition of the homogenate to the reaction medium. However, at 2

of fatty acid appeared. Inspection of Table II reveals that there is an upper limit on the rate of oxygen uptake by the heart muscle homogenate for the first 20 minute period. When fumarate concentrations of 0.01 M and above were used in several preparations, no extra oxygen uptake appeared over the 2 hour incubation period.

This factor, which may possibly be a matter of competition for hydrogen acceptors, is of itself perhaps not worthy of special note but assumed considerable practical importance in the experiments described here. For instance, when rat heart muscle suspensions were first examined for their ability to oxidize fatty acids, high concentrations of fumarate and short reaction periods were used. Under these conditions extra oxygen uptake in

TABLE II

Effect of Fumarate Concentration on Extra Oxygen Uptake with Fatty Acid

The Warburg vessels contained 1.0 ml. of homogenate, 0.20 ml. of MgSO_4 (0.005 M), 0.20 ml. of malonate (0.01 M), 0.20 ml. of adenosine triphosphate (0.0012 M), 0.10 ml. of cytochrome c (1×10^{-5} M) in phosphate buffer (0.005 M), 0.10 ml. of H_2O or octanoate (0.001 M), and 0.20 ml. of fumarate in concentrations listed below. Total volume 2.0 ml.; temperature 25°.

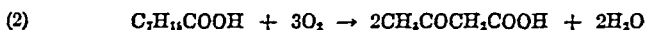
Experiment No.	Octanoate	Fumarate concentration M	Oxygen uptake	
			20 min.	120 min.
			c.mm.	c.mm.
1	—	0.001	70	100
.	+	0.001	130	142
2	—	0.002	108	176
	+	0.002	160	261
3	—	0.005	143	330
	+	0.005	167	490
4	—	0.010	162	523
	+	0.010	162	568

the presence of fatty acid did not appear and it was at first concluded that the suspensions were not able to oxidize fatty acids. This factor was also of practical importance in the experiments described in the following section in which it was desired to have the maximum *extra* oxygen uptake in the presence of fatty acid to allow a quantitative study of the reaction products. Although extra oxygen uptake in the presence of fatty acid was readily obtained in some degree in different preparations, it was usually relatively small compared to the oxygen uptake in the presence of fumarate alone. For this reason it was desirable to select for analysis and presentation in Table III only those experiments in which the extra oxygen uptake in the presence of fatty acid was equal to or at least of the

same order of magnitude as the uptake in the presence of fumarate alone to insure maximum significance in the analytical differences.

Great difficulty was experienced in preparing solutions of fatty acids containing 16 to 18 carbon atoms which would yield reproducible rates of oxidation by these preparations. This difficulty has been noted in previous work with the liver enzyme (3, 4) and again emphasizes one of the greatest problems in the study of fatty acid metabolism *in vitro*; namely, the production and maintenance of the proper colloidal conditions of the substrates and enzymes involved. The oxygen uptake of the heart muscle suspensions was greatly inhibited by high concentrations of fatty acids, resembling the liver system in this respect (4).

Products of Fatty Acid Oxidation in Heart Muscle Homogenates—In the washed liver suspension studied previously the absence of endogenous metabolic events permitted a quantitative study of the products of fatty acid oxidation (6). In the absence of the 4-carbon dicarboxylic acids, octanoate was oxidized completely to acetoacetate, according to the equation



The liver preparation was unable to oxidize acetoacetate. In the presence of oxalacetate or its precursors, less acetoacetate was formed from octanoate but extra citrate, α -ketoglutarate, and succinate accumulated. The four products accounted for at least 90 per cent of the amount of fatty acid undergoing oxidation. These studies indicated that in the liver preparation fatty acid oxidation proceeds either quantitatively to acetoacetate or in the presence of oxalacetate partly to acetoacetate and partly through the Krebs cycle, depending on the supply of oxalacetate.

With this evidence on the liver system at hand, a study was made of the products of fatty acid oxidation in heart muscle homogenates. It is well known that most extrahepatic tissues utilize acetoacetate at a high rate and it was therefore expected that the heart muscle homogenates would show little or no acetoacetate accumulation during fatty acid oxidation (if acetoacetate actually is formed). Since the Krebs cycle has been implicated in acetoacetate oxidation in certain extrahepatic tissues (8) and in fatty acid oxidation in the liver (5, 6), it appeared logical to look for those intermediates of the Krebs cycle which are expected to accumulate before the point of malonate inhibition (succinic dehydrogenase) during fatty acid oxidation in the heart muscle suspension.

In Table III are shown analytical data on oxygen uptake and the formation of acetoacetate, citrate, and α -ketoglutarate plus succinate in preparations of heart muscle acting on fatty acids in the presence of malonate under a variety of experimental conditions. In the absence of fumarate

no extra accumulation of intermediates occurred with fatty acids. With simultaneous fumarate oxidation, however, fatty acids showed extra oxygen uptake, negligible accumulation of acetoacetate or citrate, but a considerable extra accumulation of α -ketoglutarate plus succinate. There was no formation of succinate when octanoate and fumarate were incubated with the malonate-inhibited suspension anaerobically. The oxidation of fumarate alone yielded succinate, as was expected, since heart muscle is capable of catalyzing the reactions of the Krebs tricarboxylic acid cycle

TABLE III

Products of Fatty Acid Oxidation in Heart Muscle Homogenates

The Warburg vessels contained 3.0 ml. of homogenate, 0.60 ml. of MgSO_4 (0.005 M), 0.60 ml. of malonate (0.01 M), 0.60 ml. of sodium adenosine triphosphate (0.0012 M), 0.20 ml. of phosphate buffer (0.005 M), 0.20 ml. of cytochrome *c* (1×10^{-5} M), 0.40 ml. of H_2O or α -ketoglutarate (0.002 M) or fumarate (0.005 M), and 0.40 ml. of H_2O or octanoate (0.001 M) or laurate (0.0008 M). Total volume 6.0 ml.; temperature 23° ; time 90 minutes for Experiments 1, 2, and 3, 158 minutes for Experiment 4.

Experiment No.	Substrate	α -Keto-glutarate	Fumarate	O_2 uptake	Aceto-acetate formed	Citrate formed	α -Keto-glutarate + succinate formed
				<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>
1	None	—	—	164	16	9	82
	Octanoate	—	—	152	14	4	80
2	None	+	—	262	17	7	260
	Octanoate	+	—	292	21	6	279
3	None	—	+	634	24	21	292
	Octanoate	—	+	1156	20	20	512
	Laurate	—	+	1202	24	16	540
	Octanoate (anaerobic)*	—	+				31
4	None	—	—	260			128
	"	—	+	1160	20		496
	Octanoate	—	+	1860	22		910

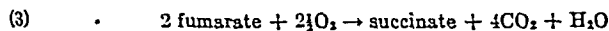
* In this experiment the gas phase was nitrogen and the substrates were added from the side arm after equilibration.

(9). When fatty acids were also present, the succinate accumulation was nearly double the values obtained with fumarate alone. This extra accumulation of succinate in the presence of fatty acid indicates that fragments formed during the oxidation of fatty acid were converted into succinate. In view of previous work of the author on the mechanism of citrate and succinate formation from fatty acids in malonate-inhibited liver suspensions (6), the formation of extra succinate from fatty acid in this situation therefore may have resulted from condensation of a 2-carbon frag-

ment arising from the fatty acid with a molecule of oxalacetate (derived from fumarate) to form tricarboxylic acid. The mechanism of formation of succinate from *cis*-aconitate, isocitrate, and citrate is of course well known (10, 11). The failure of citrate to accumulate is not surprising, since the preparation was capable of oxidizing both citrate and *cis*-aconitate at a high rate, as was determined by direct trial. The further oxidation of the succinate formed (which in these experiments was prevented by malonate) would regenerate a molecule of oxalacetate, which could then reengage in the cycle by condensation with another 2-carbon fragment arising during fatty acid oxidation.

This interpretation of the mechanism is illustrated more strikingly by the following analysis of the data on Experiment 4, Table III.

In Experiment 4, Table III, the amount of oxygen required to bring the reaction



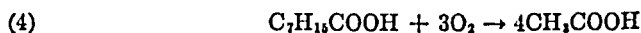
to completion is 840 c.mm. The amount of succinate formed according to this equation (assuming that succinic dehydrogenase is completely inhibited by the malonate present) is 336 c.mm. At 158 minutes (when the rate of oxygen uptake in the presence of fumarate had dropped to the rate in the flask without fumarate) the oxygen uptake in the presence of fumarate was 1160 c.mm. The endogenous uptake (without fumarate) was 260 c.mm. The oxygen uptake for fumarate alone therefore was $1160 - 260 = 900$ c.mm., or reasonably close to that expected, 840 c.mm., for the complete oxidation according to equation (3). The succinate actually formed was 496 c.mm. The endogenous formation of succinate was 128 c.mm. The succinate formed from *added* fumarate was therefore $496 - 128 = 368$ c.mm., agreeing closely with that expected (336 c.mm.) on the basis of equation (3). The analytical data are therefore in agreement with the conclusion that fumarate was oxidized to succinate completely in 158 minutes, according to equation (3).

Equation (3) may be explained by assuming that 2 moles of fumarate are oxidized to 2 of oxalacetate, 1 of which decomposes to form pyruvate. 1 mole of pyruvate then reacts with 1 of oxalacetate to produce 1 mole of tricarboxylic acid and ultimately 1 mole of succinate. Now, if there should be another source of short chain fragments capable of reacting with oxalacetate to produce tricarboxylic acid, potentially 2 moles of succinate could be formed from 2 of fumarate instead of only 1. Therefore, any increase in yield of succinate in the presence of fatty acid indicates another source of reactive fragment, i.e. the fatty acid. In Experiment 4, $910 - 496$ or 414 c.mm. of extra succinate appeared, somewhat more than the 336 c.mm. of extra succinate possible according to this interpretation.

It is probable that the endogenous respiration also furnished some oxalacetate for extra succinate formation from fatty acid to account for this figure. In view of the considerations outlined, the extra succinate formed must have been derived from fatty acid.

If each mole of extra succinate appeared by condensation of 1 mole of 2-carbon fragment derived from fatty acid with 1 mole of oxalacetate, then 414 c.mm. of 2-carbon fragments engaged in this process. This amount of 2-carbon fragments is equivalent to $414/4$ or 103 c.mm. of octanoate. At the beginning of the reaction 134.4 c.mm. of octanoate were present. The extra succinate formed therefore could account for the carbon of $103/134 \times 100$ or 77 per cent of the fatty acid originally added.

The oxidation of 134.4 c.mm. of octanoate (the amount originally present) to 4 moles of acetate (or some reactive fragment having the same oxidation level as acetate) requires 403 c.mm. of oxygen, according to the equation



The conversion of this amount of acetate into succinate (assuming condensation with oxalacetate to *cis*-aconitate and oxidation of *cis*-aconitate to succinate) requires 538 c.mm. of oxygen. The total oxygen required to convert the octanoate originally present into succinate is therefore $403 + 538 = 941$ c.mm. of O_2 . Since the yield of succinate accounted for 77 per cent of the octanoate originally present, the extra oxygen taken up, according to this interpretation, would be expected to be $0.77 \times 941 = 725$ c.mm. The extra oxygen uptake actually found was $1860 - 1160 = 700$ c.mm. It must be concluded that the fatty acid which underwent oxidation was quantitatively converted into succinate, both oxygen uptake and succinate accumulation data agreeing with this conclusion.

It would be desirable to have analytical data on the disappearance of fatty acid in this experiment to complete a carbon balance sheet. The determination of octanoate by steam distillation and titration in the amounts used in these experiments (the equivalent of 0.60 ml. of 0.01 *N* NaOH per Warburg vessel) has not been very successful in the author's hands. For instance, when 10 micromoles of octanoate were added to a heart muscle suspension, followed by immediate acidification with phosphoric acid, the recovery of octanoic acid with constant volume distillation never exceeded 8.0 micromoles. This difficulty, combined with the need for relatively large aliquots of the reaction media for the succinate determinations, as well as the necessity for selecting for analysis only those experiments in which the extra oxygen uptake in the presence of octanoate was relatively great compared to that in the presence of fumarate alone (due to considerations already outlined), precluded a complete carbon balance experiment on the same enzyme suspension. However, in a single

large scale oxidation in which a suspension prepared from seven rat hearts was allowed to act on 24 micromoles of octanoate in an experiment resembling that under discussion above, some 60 per cent of the fatty acid disappeared. This figure was corrected for a negative analytical error of 20 per cent.

Acetoacetate and Acetate Oxidation in Heart Muscle Suspensions—The data in Table III show that acetoacetate does not accumulate as a product of fatty acid oxidation in heart muscle suspensions. Previous work indicated that fatty acid was quantitatively oxidized to acetoacetate in washed liver suspensions. Intact liver has only a limited ability to oxidize acetoacetate; extrahepatic tissues such as the heart (12) can oxidize acetoacetate at a high rate. It is possible therefore that acetoacetate was

TABLE IV

Formation of α -Ketoglutarate Plus Succinate from Acetoacetate in Heart Muscle Homogenate

The Warburg vessels contained 3.0 ml. of homogenate, 0.6 ml. of MgSO_4 (0.005 M), 0.60 ml. of malonate (0.01 M), 0.60 ml. of sodium adenosine triphosphate (0.0009 M), 0.30 ml. of phosphate buffer, pH 7.4 (0.007 M), 0.30 ml. of cytochrome c (5×10^{-4} M), 0.20 ml. of fumarate (0.0025 M), and 0.40 ml. of H_2O or sodium acetate (0.004 M) or sodium acetoacetate (0.002 M). Total volume 6.0 ml.; temperature 25° ; time 70 minutes.

Substrate	O_2 uptake	Change in acetoacetate	Formation of α -ketoglutarate plus succinate
	c.mm.	c.mm.	c.mm.
None.....	421	+4	192
Acetate.....	502	+8	198
Acetoacetate (269 c.mm.)....	883	-78	328

the primary product of oxidation of octanoate in the experiments recorded in Table III, but that it was oxidized further (via the Krebs cycle) at a rate at least as great as its rate of formation from octanoate. The rate of acetoacetate oxidation by the heart muscle preparation was therefore determined. Since acetate (or some labile, reactive metabolite to which it may be converted enzymatically) has been implicated in acetoacetate metabolism recently (8, 13, 14) the rate of oxidation of acetate was also measured. Fumarate was added in all experiments. Oxygen consumption, acetoacetate formation or destruction, and succinate formation were measured. All conditions were comparable to those of Table III, with the exception that the incubation period was shorter and the fumarate concentration lower.

It can be seen from the typical data in Table IV that acetoacetate gives a considerable extra oxygen uptake. At the same time there were a dis-

appearance of acetoacetate and formation of extra succinate in such amounts as to account nearly quantitatively for the acetoacetate which disappeared.

Acetate formed only traces of acetoacetate and succinate. Acetate was therefore probably not an intermediate in the oxidation of acetoacetate.

It is evident from consideration of these data that acetoacetate could conceivably have been a primary product of fatty acid oxidation in the experiments of Table III, since the rate of oxidation and the rate of succinate formation appear to be of the same magnitude as the rate required by the kinetics of fatty acid oxidation. Under these conditions no acetoacetate would be expected to accumulate if it were an intermediate in fatty acid oxidation.

DISCUSSION

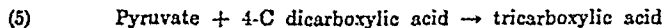
The evidence presented in this paper shows that an extrahepatic tissue, rat heart muscle, possesses the enzymatic equipment not only for the oxidation of acetoacetate but also for the direct oxidation of fatty acids. This evidence does not, of course, indicate under what conditions fatty acids are oxidized by heart muscle *in vivo*.

The fatty acid oxidation by heart muscle resembles that in liver suspensions in that it requires adenine nucleotide and causes succinate accumulation in the presence of malonate, when fumarate is oxidized simultaneously. There are several points of difference. In the liver suspension, ATP causes fatty acid oxidation by itself, leading to acetoacetate formation. This does not appear to occur in the heart muscle preparation. The oxidation of α -ketoglutarate to succinate is capable of causing the oxidation of fatty acids in liver, but is incapable of activating fatty acid oxidation in the heart muscle suspension.

The requirement of fumarate in activating fatty acid oxidation in the heart muscle suspensions appears to be specific; in several experiments oxalacetate did not produce the same effect. Previous work (4, 6) on the liver enzyme system has indicated *two* separate factors in the need for fumarate: (a) the generation of high energy phosphate bonds (but not necessarily those of ATP) during fumarate oxidation which are capable of "activating" the fatty acid oxidation and (b) the formation of oxalacetate for condensation with a fragment released from the fatty acid after oxidation to form tricarboxylic acid.

There is as yet not enough information at hand to speculate further on the nature of the activation of fatty acid oxidation, on the details of the oxidation mechanism, and on the mechanism of the integration with the Krebs tricarboxylic acid cycle. Indeed, although the over-all mechanism

of the Krebs cycle appears well established, little is known concerning the reaction by which tricarboxylic acid is formed; i.e., the over-all reaction



As Krebs has pointed out in his recent review (15), it is possible that pyruvate is first oxidized to a 2-carbon fragment, which then might react with oxalacetate to form a 6-carbon tricarboxylic acid directly. The possible participation of phosphorylated intermediates in the condensation reaction cannot be overlooked. Since this condensation reaction appears to be the connecting link between fatty acid or acetoacetate oxidation and the Krebs cycle in view of recent work by several groups of investigators, study of this reaction should be particularly fruitful in the future.

Krebs and Eggleston have taken exception to the possible rôle of the tricarboxylic acid cycle in acetoacetate metabolism (16, 17). They point out that the effect of oxalacetate in accelerating acetoacetate disappearance and in the formation of extra citrate noted by Breusch (12) and Wieland and Rosenthal (18) can be explained by postulating the occurrence of a series of known oxidation reductions which lead to the reduction of acetoacetate to β -hydroxybutyrate (accounting for acetoacetate disappearance) and the formation of extra citrate by reactions consequent to the reduction of acetoacetate. By following the variation in concentration of some fifteen intermediates concerned in these dismutations after incubation of oxalacetate and acetoacetate *anaerobically* with sheep heart muscle suspensions, they have concluded that all of the acetoacetate which disappeared could be recovered as β -hydroxybutyrate and that the extra citrate which appeared was not derived from the acetoacetate molecule. However, Buchanan *et al.* (8) have decisively proved that acetoacetate enters into the formation of members of the tricarboxylic acid cycle under *aerobic* conditions, since isotope (C^{13}) of labeled acetoacetate was incorporated into α -ketoglutarate, succinate, and fumarate in kidney homogenates *aerobically* in such amounts as to demonstrate a major pathway for the oxidative degradation of acetoacetate (and acetate). The author has also presented data on citrate formation from fatty acids in liver suspensions (6) which cannot be explained on the basis which Krebs and Eggleston have proposed, since in this situation acetoacetate does not disappear in the presence of oxalacetate and no extra citrate is formed. A *precursor* of acetoacetate appears to be involved in citrate formation in this case.

There are indications that the work of Krebs and Eggleston is not necessarily inconsistent with the findings of Buchanan *et al.* Krebs and Eggleston point out that malonate inhibits acetoacetate removal very strongly under *aerobic* conditions, whereas it has no effect *anaerobically*. It is

probable that the anaerobic experiments of Krebs and Eggleston do not deal with the same system which caused the effects noted by Buchanan *et al.* The forthcoming report by Krebs and Eggleston on the aerobic metabolism of acetoacetate will therefore be awaited with great interest.

Several other extrahepatic tissues of the rat have been examined for their ability to oxidize higher fatty acids under conditions resembling those outlined in this paper. Kidney and skeletal muscle preparations have also given indications of ability to oxidize fatty acids, although it was found that the conditions of homogenization were very critical in obtaining active preparations. In view of such difficulties and the factors already outlined in this paper, it does not appear profitable at this time to extend work on the extrahepatic tissues until some of the individual enzymes and intermediates concerned in fatty acid oxidation have been identified, for which purpose the liver system appears best adapted. It is evident that the reactions described are complex and highly organized, and for this reason it is not yet possible to assay different tissues for their ability to oxidize fatty acids on a quantitative basis.

SUMMARY

Rat heart muscle suspensions are capable of the oxidation of higher saturated fatty acids, a reaction which requires the presence of adenine nucleotide and simultaneous fumarate oxidation. Extra succinic acid accumulates as the end-product of fatty acid oxidation in these preparations when succinic dehydrogenase is inhibited by malonate. Analytical data show that the extra succinate which accumulates accounts quantitatively for the fatty acid oxidized if it is assumed that 2-carbon fragments from the fatty acid combine with oxalacetate to form tricarboxylic acid and ultimately succinate. Citrate and acetoacetate do not accumulate during the oxidation of the fatty acid. Acetoacetate, however, is readily oxidized by the preparation with the formation of extra succinate. Acetate forms neither acetoacetate nor succinate.

The results strongly suggest that both fatty acid oxidation and acetoacetate oxidation proceed through the Krebs tricarboxylic acid cycle in heart muscle suspensions.

The author wishes to acknowledge the technical assistance of Mrs. D. E. Smith in this investigation.

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STUDIES IN CARBOHYDRATE METABOLISM

VII. THE DISTRIBUTION OF DEUTERIUM IN A SAMPLE OF DEUTERIO GLUCOSE EXCRETED BY A DIABETIC RABBIT*

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When glucose or glycogen is isolated from the body of an animal whose body fluids have been enriched with D_2O , deuterium is invariably found stably bound in the product isolated. As any isotope which may have been present in the hydroxyl hydrogen of such a sample will have been washed out and lost in the process of isolation, the deuterium with which we are here concerned may be assumed to be bound to carbon, and the introduction of such carbon-bound deuterium is apparently the result of synthetic processes in the animal. Our reasons for rejecting the possibility of the biological introduction of deuterium into the carbon-bound positions of glucose consequent to labilization and exchange of these hydrogens have been stated in an earlier communication (1).

In such a sample of deuterio glucose, isolated from an animal whose body fluids contained D_2O , it was considered of interest to investigate the intimate distribution of the deuterium among the hydrogen atoms bound to carbon in glucose. In a similar study, the distribution of isotopic carbon among the 6 carbon atoms of glucose synthesized from various isotopic precursors has been investigated by Lorber, Lifson, and Wood (2). The methods employed by these authors, a combination of chemical and bacteriological degradations, were well suited to their problem, but are obviously completely unsuited for a study of the location of deuterium atoms in glucose.

In order to estimate the isotopic composition of the hydrogen attached to each of the carbon atoms of glucose, it is necessary to prepare a series of derivatives such that one or more of the hydrogen atoms in question is eliminated. The starting material, in the present instance, was a pooled urinary glucose sample, excreted over a period of 48 hours by a rabbit rendered diabetic with alloxan (3). During this period, the rabbit was consuming *ad libitum* a diet containing 62 per cent of carbohydrate and its body fluids contained 1.05 to 1.09 atom per cent excess of D. The urinary glucose was isolated as the pentaacetate, and this product contained

* This work was carried out with the aid of grants from the Josiah Macy, Jr., Foundation and the Nutrition Foundation, Inc.

gen in the original glucose (column (e), Table I). It is clear that if the isotope were uniformly distributed among the 7 carbon-bound hydrogen atoms of the original glucose, this calculation would be expected to give the same answer in each case.

Finally, on the basis of the analytical values, it is possible to calculate the concentrations of isotope at each of the individual positions. As the values obtained in the succeeding calculations represent, in each case, relatively small differences between large numbers, analytical errors will

TABLE I

Concentration of Deuterium in Products Obtained from Deuterio Glucose

The concentration of deuterium found in each of the derivatives has been compared with the value predicted on the assumption of uniform distribution of isotope among the carbon-bound hydrogens. The average D concentration among the surviving glucose H atoms in each derivative has also been calculated.

Compound	Total No. of H atoms	No. of H atoms initially bound to C of glucose	Positions of glucose from which H has been eliminated	Calculated D concentration in derivatives	Analysis, D concentration in derivatives	Average D concentration in stable H atoms remaining from glucose
	(a)	(b)		(c)*	(d)	(e)†
				atoms per cent	atoms per cent	atoms per cent
Glucose pentaacetate	22	7			0.159	0.500
Potassium gluconate	11	6	I	0.273	0.261	0.479
Gluco-benzimidazole	16	6	I	0.188	0.190	0.507
Glucosazone	22	6	II	0.136	0.135	0.495
Potassium acid saccharate	9	4	I, VI, VI	0.222	0.250	0.563
Silver saccharate	8	4	I, VI, VI	0.250	0.287	0.574
Trimethoxyglutaramide	16	3	I, V, VI, VI	0.094	0.094	0.500
Dimethoxysuccinamide	12	2	I, IV, V, VI, VI	0.083	0.081	0.486

* Calculated on the assumption of the uniform distribution of D among the 7 stable hydrogens of glucose, $c = 0.500 b/a$.

† $e = ad/b$.

necessarily become magnified and the values can therefore not be expected to be very precise. The deuterium concentrations, computed for each position, are as follows:

Position I, by comparing the pentaacetate with the benzimidazole

$$7 \times 0.500 = 6 \times 0.507 + D_I$$

$$D_I = 0.46 \text{ atom per cent}$$

Position II, by comparing the pentaacetate with the osazone

$$7 \times 0.500 = 6 \times 0.495 + D_{II}$$

$$D_{II} = 0.53 \text{ atom per cent}$$

Position III, by comparing dimethoxysuccinamide with D_{II}

$$2 \times 0.486 = 0.53 + D_{III}$$

$$D_{III} = 0.44 \text{ atom per cent}$$

Position IV, by comparing trimethoxyglutaramide with dimethoxysuccinamide

$$3 \times 0.500 = 2 \times 0.486 + D_{IV}$$

$$D_{IV} = 0.53 \text{ atom per cent}$$

Position V, by comparing potassium acid saccharate with trimethoxyglutaramide

$$4 \times 0.563 = 3 \times 0.500 + D_V$$

$$D_V = 0.75 \text{ atom per cent}$$

Position VI, by comparing the benzimidazole with the saccharate

$$6 \times 0.507 = 4 \times 0.563 + 2D_{VI}$$

$$D_{VI} = 0.40 \text{ atom per cent}$$

A complete formulation of this deuterio glucose sample is given in Fig. 2. All of these calculations are predicated upon the assumption that no isotope was lost by labilization and exchange in the course of the preparation of the several derivatives. This belief finds support in the fact that the average of the concentrations of isotope in the 7 carbon-bound hydrogen atoms, as calculated from the individual values,

$$\frac{0.46 + 0.53 + 0.44 + 0.53 + 0.75 + 2 \times 0.40}{7} = 0.50 \text{ atom \%}$$

is in agreement with the value of 0.500 based on the original analysis⁵ of glucose pentaacetate.

From this it appears that there is isotope present among the hydrogen atoms that are bound to each of the 6 carbon atoms of the glucose sample under investigation. Furthermore, it is apparent that the concentrations of D in each of these positions are of the same order of magnitude. The value which deviates most from the average is that at position V. This deviation results from the finding of a higher concentration of isotope in potassium acid saccharate than was predicted on the assumption of uniform distribution (Table I) and is, we believe, somewhat greater than would be anticipated on the basis of experimental error. Two separate preparations of potassium acid saccharate gave analytical values for D that were in perfect agreement, and the analysis of silver saccharate also confirms this finding. However, in view of the fact that this study was limited to a single preparation of deuterio glucose isolated from the urine of a single animal, this finding is not at present susceptible of interpretation.

The finding that D_{II} is not significantly higher than the average D concentration of the carbon-bound hydrogens of this sample of glucose militates against the hypothesis that rapid, reversible enolization of glucose occurs in the animal. Such a reaction, if it did occur, would necessarily result in the introduction of isotope at position II, and the concentration of deuterium at this position would soon approach that of the aqueous en-

vironment which, in the present instance, was above 1 per cent. Because they failed to find any stably bound D in the reaction products when glucose underwent mutarotation or transformation to fructose in a medium of D_2O , Fredenhagen and Bonhoeffer eliminated the enediol of glucose as an obligatory intermediate in either of these reactions *in vitro* (5). The present evidence would seem to eliminate reversible enediol formation as a major process in glucose metabolism.

The most plausible explanation for the appearance of deuterium in a sample of glucose excreted in the urine of an animal whose body fluids contain an excess of D_2O is that a portion of this glucose has been synthesized from fragments such that each hydrogen atom in the glucose ultimately formed either arose from or was at some time in equilibrium with the body fluid. In earlier studies (3, 6) we have estimated the proportion of urinary

Deuterium conc.			% of body water
I	0.46	$D-C \begin{array}{c} \text{OH} \\ \\ \text{---} \end{array}$	43
II	0.53	$D-C \begin{array}{c} \text{OH} \\ \\ \text{---} \end{array}$	50
III	0.44	$HO-C \begin{array}{c} \text{D} \\ \\ \text{---} \end{array}$	41
IV	0.53	$D-C \begin{array}{c} \text{OH} \\ \\ \text{---} \end{array}$	50
V	0.75	$D-C \begin{array}{c} \text{---} \\ \\ \text{---} \end{array}$	70
VI	0.40	$\left\{ \begin{array}{c} D-C \begin{array}{c} \text{OH} \\ \\ \text{---} \end{array} \\ D \end{array} \right.$	37

FIG. 2. The intimate isotopic constitution of the present sample of glucose. The concentration of D at each stable position is given both as the actual value, in atom per cent excess, and as the per cent of D present in the body water of the rabbit.

glucose synthesized *in vivo* on the assumption that the urinary glucose is composed of two fractions, the first derived from the diet and devoid of stably bound deuterium, the second synthesized by the animal and containing, in its carbon-bound hydrogen, isotope derived from the body fluid. The present findings that deuterium is present in the hydrogen attached to each carbon atom of the glucose in question and that the concentration of isotope is of the same magnitude at each position are in accord with the foregoing assumption. It may therefore be concluded that in the present experiment, in which the concentration of D in the body water was 1.05 to 1.09 atom per cent and the average concentration of D among the stable hydrogen atoms of glucose was 0.50 atom per cent, about one-half of the glucose excreted was synthesized *in vivo*, the remainder presumably arising from the diet. If the synthesized glucose and dietary glucose are

pictured as undergoing perfect mixing in the animal and if the urinary glucose is taken as a true sample of this mixture, it follows that the quantity of glucose synthesized in this rabbit per day was roughly equal to the quantity ingested.

EXPERIMENTAL

The glucose which served as the starting material in the present investigation was excreted over a period of 48 hours in the urine of a 2 kilo female rabbit which had been rendered diabetic with alloxan (3). During this period the rabbit consumed daily from 150 to 200 gm. of a diet containing 62 per cent of carbohydrate, and on the 2 days of the experiment excreted 52.2 and 54.0 gm. of glucose. The body fluids, initially enriched with D_2O by intravenous injection and maintained by replacement of the drinking water by an appropriate concentration of D_2O , contained 1.05 and 1.09 atom per cent excess D on the 2 experimental days. The glucose from each day's urine was isolated as the pentaacetate, the sample from the 1st day containing 0.144, that from the 2nd day 0.166 atom per cent D. These samples were combined and once recrystallized from ethanol-water and the 106 gm. of nearly colorless crystals were employed in the preparation of the following products.

Glucose Pentaacetate—For analysis, a sample of the above crude pentaacetate was twice recrystallized from water and dried over P_2O_5 at 100° *in vacuo*.

Calculated. C 49.2, H 5.6

Found.¹ " 49.2, " 5.5, D 0.159 atom % excess

Potassium Gluconate—4.34 gm. of glucose pentaacetate were treated with I_2 in methanolic KOH (7) and of the 2.12 gm. of crude potassium gluconate obtained 1.12 gm. were recrystallized from aqueous methanol. The product, dried *in vacuo* over P_2O_5 at 61° , weighed 0.873 gm.

Calculated. C 30.8, H 4.7

Found. " 30.9, " 4.9, D 0.261 atom % excess

Gluco-benzimidazole—The remaining gm. of potassium gluconate was condensed with *o*-phenylenediamine (7) and the resultant benzimidazole recrystallized from water. It was dried over P_2O_5 *in vacuo* at 100° and weighed 0.467 gm.

Calculated. C 53.7, H 6.0

Found. " 53.6, " 6.0, D 0.190 atom % excess

¹ Elementary microanalyses reported in this paper were performed by Mr. William Saschek.

Phenylglucosazone—2.5 gm. of recrystallized glucose pentaacetate were refluxed for 3 hours with 40 cc. of 0.3 N H_2SO_4 . 20 gm. of crystalline sodium acetate, 4.2 of phenylhydrazine hydrochloride, and 1.7 cc. of a saturated aqueous solution of NaHSO_3 were then added and the mixture heated for 3 hours on the steam bath. After prolonged cooling, the precipitate was filtered off and recrystallized from pyridine-benzene. The product was dried *in vacuo* successively over H_2SO_4 at room temperature and over P_2O_5 at 61° and weighed 1.377 gm.

Calculated. C 60.3, H 6.2

Found. " 60.5, " 6.0, D 0.135 atom % excess

2,3,4,6-Tetramethylglucose—30 gm. of glucose pentaacetate were refluxed for 3 hours with 400 cc. of 0.3 N H_2SO_4 . The solution was cooled and brought almost to neutrality by the addition of NaOH solution and was then concentrated *in vacuo* to a volume of 25 cc. Methylation was carried out in this solution according to the procedure of West and Holden (8) with the single alteration that the quantities of both dimethyl sulfate and NaOH were increased by a factor of 33 per cent. The product was recrystallized by mixing with anhydrous CaSO_4 , and continuously extracting the resultant powder in a Soxhlet apparatus with petroleum ether to which had been added 0.5 per cent of ether. 4.7 gm. of white crystals were obtained.

d-Dimethoxysuccinamide—4 gm. of the above tetramethylglucose were oxidized with HNO_3 (sp. gr., 1.42), according to the procedure employed by Hirst (4). After esterification with methyl alcohol, the products of oxidation were distilled at a pressure of 2 mm. and the middle fraction, 2.5 gm. of a pale yellow oil, distilling between $135\text{--}140^\circ$, were treated with dry NH_3 in methanol. The crystals which separated on refrigeration were collected and a second crop was obtained, after isolation of the xylo-trimethoxyglutaramide (see below) by concentration of the mother liquor and resaturation with NH_3 . d-Dimethoxysuccinamide was recrystallized from hot water and dried at 61° *in vacuo* over P_2O_5 . The product weighed 255 mg. and melted with decomposition at $270\text{--}280^\circ$.

Calculated. C 40.9, H 6.8

Found. " 41.4, " 6.8, D 0.081 atom % excess

Xylotrimethoxyglutaramide—After the mother liquor of the first precipitation of the foregoing dimethoxysuccinamide had been concentrated under reduced pressure and set in the refrigerator, this compound precipitated. It was recrystallized from a hot 1:1 mixture of ethanol and ether and dried over P_2O_5 *in vacuo* at 61° ; the product weighed 361 mg. and melted at 195° .

Calculated. C 43.6, H 7.3

Found. " 43.5, " 7.0, D 0.094 atom % excess

Potassium Acid Saccharate—4.4 gm. of potassium gluconate, prepared from 10 gm. of the pentaacetylglucose, were added with cooling to 12 cc. of concentrated HNO_3 (sp. gr. 1.4) (9). Reaction was initiated by addition of 10 drops of fuming HNO_3 and the mixture allowed to stand for 2 days at room temperature. The mixture was made slightly alkaline with K_2CO_3 , then acidified with acetic acid, and refrigerated for several days. The resulting precipitate was recrystallized twice from a small volume of hot water. The 0.596 gm. obtained was dried at 61° *in vacuo* over P_2O_5 .

Calculated. C 29.0, H 3.6

Found. " 29.2, " 3.7, D 0.250 atom % excess

Starting with another batch of glucose pentaacetate, we made a second preparation of potassium acid saccharate by the same method.

Found. D 0.250 atom % excess

Silver Saccharate—1.66 gm. of potassium acid saccharate were converted to silver saccharate by the method of Gans and Tollens (10) and the 0.712 gm. obtained was dried at room temperature *in vacuo* over H_2SO_4 .

Calculated. C 17.0, H 1.9, Ag 50.9

Found. " 16.8, " 2.0, " 50.9, D 0.290 atom % excess

A second sample of silver saccharate was made by the same method.

Found. D 0.284 atom % excess

SUMMARY

A sample of deuterio glucose excreted by a diabetic rabbit whose body fluids were enriched with D_2O has been studied in order to ascertain the concentration of deuterium among the carbon-bound hydrogen atoms at each position. Deuterium has been demonstrated to be present at each position, and its concentration at each of the several positions has been found to be of the same order of magnitude. The only significant deviation from the average value was at position V, where a somewhat higher concentration of D was found than at the other positions. The results rule out reversible enolization of glucose as a major metabolic pathway and support the view that the concentration of D in a sample of urinary glucose, excreted by an animal receiving D_2O , is a measure of the amount of glucose synthesized.

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STUDIES IN CARBOHYDRATE METABOLISM

VIII. THE ORIGIN OF THE STABLE HYDROGEN IN GLYCOGEN FORMED FROM VARIOUS PRECURSORS*

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If in the course of a synthetic operation in the animal, hydrogen originating in the body water enters into stable organic linkage, the process is susceptible of study by observation of the amount of deuterium introduced when the fluids of the animal's body are enriched with D_2O . This technique has been employed to investigate the rate of synthesis of fatty acids and cholesterol (1), and more recently we have employed this procedure to study the rate of glycogen formation in the experimental animal (2).

In the course of this study it was early apparent that in the interpretation of the quantity of deuterium present in a given sample of glycogen it was necessary to consider not only the rate of its formation but also the nature of the precursors from which it arose. Thus it has been reported that, under our experimental conditions, the glycogen arising in the liver of previously fasted rats after feeding of lactic acid was considerably richer in deuterium than the sample obtained when glucose was fed (3). We have also reported that the glycogen that is deposited both in muscle and in liver when galactose rather than glucose comprises the bulk of the diet is not appreciably richer in deuterium than when our stock glucose diet was fed (4). The simplest explanation of these effects is that the more directly the dietary test substance is used for glycogenesis, the less opportunity there will be for the introduction of deuterium from the body fluids. If, on the other hand, before glycogen can be formed and deposited, the dietary precursor must pass through stages at which double bonds must be hydrated or hydrogenated, or at which carbon-bound hydrogens become exchangeable by enolization or other reaction, opportunity for the introduction of deuterium from the body fluid will arise, and glycogen rich in deuterium will result.

In order to gain some information as to the stages in glycogenesis at which deuterium is introduced from the D_2O of the body water, we have carried out a series of comparable experiments in which rats have been first

* This work was carried out with the aid of grants from the Josiah Macy, Jr., Foundation and the Nutrition Foundation, Inc.

fasted for 24 hours, then given an injection of D_2O , followed, after an hour, by the administration by stomach tube of one of several known precursors of glycogen. After 3 hours the animals were killed, glycogen was isolated from the livers, and its deuterium concentration compared with that of the body water.

When glucose was the test substance fed (3), the glycogen isolated from the liver was found to contain 38 per cent as much deuterium as the body water (Table I). Since, after 24 hours of fasting, the rat liver is practically devoid of glycogen, it is fair to assume that almost all of the glycogen found in the liver at the termination of such an experiment was formed after the

TABLE I
Deuterium Concentration in Liver Glycogen

Six adult female rats in each group were fasted for 24 hours and then given D_2O subcutaneously. 1 hour later the test substance indicated was fed by stomach tube in the dosage indicated in the text. 3 hours thereafter the rats were killed, glycogen isolated from their livers, and its deuterium concentration compared with that of the body water. Weights of samples are expressed as per cent of liver.

Substance fed	Body water	Liver glycogen		
		Weight	D	D
	<i>atom per cent D</i>	<i>per cent</i>	<i>atom per cent</i>	<i>per cent of body water</i>
Glucose*	1.29	0.8	0.49	38
Fructose	1.31	0.9	0.570	44
Mannose	1.35	0.1	0.471	35
Galactose	1.27	0.7	0.425	33
<i>dl</i> -Glyceraldehyde	1.28	0.6	0.552	43
<i>dl</i> -Glyceric acid	1.31	0.7	0.568	43
Dihydroxyacetone	1.33	0.3	0.747	56
<i>dl</i> -Lactic acid*	1.28	0.2	0.73	57

* Boxer and Stetten (3).

test substance had been administered and was minimally diluted by pre-existing glycogen. Its isotope content should therefore closely approach that of the glycogen formed during the test period.

It has been shown experimentally that 34 per cent of all of the hydrogen in glycogen is immediately and completely exchangeable with the hydrogen of water (2), this fraction corresponding roughly to the hydroxyl hydrogen. Any deuterium which may have been present in these exchangeable positions of glycogen during the life of the animal must have been lost during the isolation and purification of the glycogen. We therefore concluded that, of the hydrogens in glycogen, that portion over and above the sum of the 38 per cent arising from the body water and the 34 per cent that

was lost by exchange during isolation must have come from hydrogen atoms stably attached to the glucose fed, and persevering in their attachment through all the steps of glycogenesis.

Confirmation of this conclusion was not possible at that time because stably labeled deuterio glucose was not available. We have now obtained a limited amount of deuterio glucose, isolating it from the urine of diabetic rabbits whose body fluids were enriched with D_2O (5). The isotope in such samples of glucose is approximately uniformly distributed among the seven stable positions (6). The present sample of glucose contained 0.242 atom per cent D, and, as this was all present in carbon-bound positions, the average isotope concentration in these positions may be calculated as $0.242 \times 12/7 = 0.415$ atom per cent D. We have fed this material to previously fasted rats in an experiment completely parallel with the earlier glucose feeding, except that in the present instance no D_2O was injected. The glycogen isolated from the livers of these rats 3 hours later contained 0.129 atom per cent D, showing that $0.129/0.415 \times 100 = 31$ per cent of the hydrogen in the glycogen formed under these conditions arose from the stably bound hydrogen of the glucose that had been fed.

Three fractions of hydrogen in glycogen must therefore be considered. Some 34 per cent is exchangeable, and any deuterium initially present in these positions will be lost during isolation. About 38 per cent of the hydrogen in liver glycogen has been shown to arise from the body water, when glucose serves as precursor, and the deuterium upon which this conclusion is based may be assumed to be approximately uniformly distributed among the non-exchangeable positions (6). 31 per cent of the hydrogen in glycogen formed in the liver of the fasted rat when glucose is fed has now been shown to come directly from the carbon-bound hydrogen of glucose. The sum of these three fractions, each determined independently, comes to 103 per cent, which we regard as a satisfactory check.

The non-exchangeable hydrogen of glycogen is thus composed of two complementary quantities, that portion which arises from the body water and that portion which arises from stable hydrogen of the precursor. These two quantities may be evaluated by complementary experimental procedures; namely, by labeling the hydrogen of the water with D by the injection of D_2O , or by labeling the hydrogen of the precursor by its preparation in such a fashion as to introduce stably bound D. As essentially the same information is obtained by both procedures, and as the first procedure is in general simpler to carry out, this has been the technique employed in the remaining experiments to be described.

The alkali-catalyzed transformation of glucose to fructose has been shown to proceed *in vitro* in a medium of D_2O without any stably bound D appearing in the product (7). This experiment was interpreted as excluding the

enediol as a necessary intermediate in this type of transformation. It is of interest that in the present series, when the rat makes liver glycogen from fructose or mannose, it does so without introduction of appreciably more deuterium from the fluid environment than occurred when glucose served as precursor (Table I). From this we conclude that transformation of fructose or mannose to the glucose configuration in the animal is not accompanied by labilization of much of the carbon-bound hydrogen. While it is not possible to rule out the enediol of glucose as an intermediate from the figures here presented, we have other evidence which makes it appear that enolization of glucose is not a prominent reaction in the animal body (6).

Whereas it is easy to visualize the transformation of fructose or mannose into glucose, the conversion of galactose to glucose would appear necessarily to be a more complex process. Evidence has previously been presented to show that when galactose serves as a precursor for glycogen in an animal receiving D_2O , the glycogen formed contains no more deuterium than when glucose is fed (4). This observation is confirmed by the present finding, in which, after galactose was fed to previously fasted rats, the liver glycogen contained 33 per cent as high a concentration of D as did the body water. Whatever the steps in the transformation of galactose to the glucose configuration, they are not associated with extensive labilization of carbon-bound hydrogen atoms.

When this characteristic of the conversion of galactose to glycogen was first detected, several possible pathways were considered. The possibility of an intermediate cyclohexitol, such as *l*-inositol suggested by Fischer (8), has been considered, and indeed the occurrence of the biological conversion of *meso*-inositol to glucose has been demonstrated (9). From this particular isomer, however, glucogenesis seems to proceed quite slowly.

A more plausible pathway is the cleavage of galactose to two triose fragments and their immediate recondensation to give glucose. The formation of hexose *in vitro* by the aldolization of two triose fragments has been shown to proceed in D_2O without the appearance of stably bound deuterium in the product (10). Something of the same sort seems to occur in animals when glyceraldehyde serves as the precursor of glycogen. The glycogen deposited in the liver under these circumstances is not much richer in D than when glucose was fed. Both glyceraldehyde and glyceric acid are, during their incorporation into glycogen, capable of retaining many of their carbon-bound hydrogen atoms.

It is noteworthy that dihydroxyacetone behaved quite differently. When glycogen was formed from this substance, its isotope concentration was practically identical with that which was formed from lactic acid. 56 per cent of all of the hydrogen in the glycogen formed when dihydroxyacetone was fed arose from the body fluids, as compared with 57 per cent for

the analogous figure when lactic acid served as precursor. When to either of these values is added the 34 per cent of known exchangeable hydrogen, it is apparent that at most a very small fraction of the stable hydrogen in the glycogen could have arisen from the test substance. During glycogenesis from either of these precursors, practically every hydrogen atom must either have arisen from or been at some stage exchangeable with the hydrogen of the fluid environment.

This difference in behavior between glyceraldehyde and dihydroxyacetone is surprising in view of the supposed interconvertibility of the phosphoric acid esters of these two compounds in the animal (11). It should be pointed out that, whereas the triose phosphates are commonly postulated intermediates in carbohydrate metabolism, the free trioses, which were the substances administered in the present experiment, are not generally so considered. Any hypothesis offered to explain the present difference between glyceraldehyde and dihydroxyacetone should take into account the previously mentioned uniformity of distribution of deuterium among the stable positions of glucose when glucogenesis occurs in an animal on a normal diet receiving D_2O (6). The present finding suggests that, regardless of the biological interconvertibility of the triose phosphates, glyceraldehyde is not, under our experimental conditions, rapidly converted into dihydroxyacetone. To account for the more or less complete introduction of isotope into the glycogen arising from dihydroxyacetone, two possible explanations are offered. Either this compound undergoes enolization, and consequent labilization of all of its hydrogen atoms, more readily than glyceraldehyde, or the pathway from dihydroxyacetone to glycogen is more indirect than that from glyceraldehyde to glycogen.

EXPERIMENTAL

Deuterio glucose was obtained from a sample of deuterio glucose pentaacetate which had been isolated from the urine of a diabetic rabbit receiving D_2O (5). A sample of pentaacetate containing 0.132 atom per cent D was boiled under reflux in 0.5 N HCl for 3 hours; the product was taken to dryness under reduced pressure and repeatedly evaporated from water to remove volatile acids. The residue, after treatment with norit, was dissolved in sufficient water to bring the glucose concentration, determined analytically, to 15 per cent. The calculated concentration of deuterium in the glucose was 0.242 atom per cent.

Three female rats, averaging 184 gm. in weight, were fasted for 25 hours and then given deuterio glucose by stomach tube, 200 mg. per 100 gm. of body weight. 3 hours later they were killed and glycogen was isolated from their livers. The body fluids of these animals contained 0.017 atom per cent D.

Of the other test substances used, galactose, fructose, and mannose were the commercially available c.p. preparations. A sample of *dl*-glyceraldehyde was generously contributed by Professor H. O. L. Fischer, to whom the authors are deeply indebted. *dl*-Glyceric acid was prepared by the HNO_3 oxidation of glycerol, according to Kiliani (12), and was isolated as its Ca salt. It was administered as an equimolar mixture of glyceric acid and Na glycerate, prepared by treatment of Caglycerate with appropriate quantities of oxalic acid and Na oxalate. The precipitated Ca oxalate was removed by centrifugation, and the volume of the clear supernatant adjusted to give a suitable concentration of solute. Dihydroxyacetone was purchased from the Pfanstiehl Chemical Company.

In each of the experiments given in Table I, six female rats, averaging 130 to 150 gm. each, were first fasted for 24 hours. Each rat then received an injection of 99.5 per cent D_2O containing 0.9 per cent NaCl, 1 cc. per 100 gm. of body weight. 1 hour later the test substance was administered by stomach tube. The hexoses and glyceric acid were given in the dosage of 200 mg. per 100 gm. of body weight, glyceraldehyde and dihydroxyacetone, 300 mg. per 100 gm. of body weight. Incident to the tube feeding, one rat of the dihydroxyacetone group died and had to be discarded.

The isolations of glycogen from liver were conducted by the same methods as have been previously described (2).

SUMMARY

When glycogen is formed from administered glucose in the liver of a rat, a portion of its carbon-bound hydrogen arises from the stable hydrogen of the glucose fed, the remaining complement coming from the body water.

When fructose, mannose, galactose, glyceraldehyde, or glyceric acid serves as dietary precursor, the complement of hydrogen in glycogen arising from the body water is not significantly increased.

During glycogenesis from dihydroxyacetone, as from lactic acid, essentially all of the hydrogen in glycogen arises from the body water.

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DIETARY PROTEIN AND PROTOPORPHYRIN FORMATION IN THE RAT*

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The wide-spread occurrence of the porphyrins as constituents of many important respiratory catalysts, such as hemoglobin, the cytochromes, and catalase, attaches fundamental importance to the problem of the origin and metabolism of these substances in the organism. Progress in this field has been retarded by a lack of satisfactory methods of quantitative analysis. However, in 1943, Grinstein and Watson (1) published a colorimetric method for the determination in erythrocytes of protoporphyrin, an alleged intermediate in the formation of heme. The introduction of this method appeared to offer an opportunity for a quantitative study of protoporphyrin in other body tissues and in the excreta.

Earlier work by other investigators has demonstrated that protoporphyrin is the predominant porphyrin appearing in the feces of the rat and that only a trace is eliminated in the urine (2, 3), even after the parenteral administration of protoporphyrin (3). Little is present in other body fluids or tissues with the exception of the Harderian glands (4), the intestinal contents (4), erythrocytes, especially reticulocytes (1), and in the white matter of the central nervous system (5). The fecal protoporphyrin appears to be that formed in excess of the requirement for hemoglobin synthesis and excreted in the feces perhaps by way of the Harderian glands in the rat, since the removal of these structures results in a marked decrease in fecal protoporphyrin to practically nil (4). Some protoporphyrin may be synthesized by the bacteria of the intestinal flora, although the amount from this source appears to be extremely small (4, 6) unless there is hemorrhage into the intestine (7).

In view of the above evidence, the assumption that protoporphyrin synthesis in the rat could be ascertained from fecal protoporphyrin excretion appears justified. The present study was designed, therefore, to determine whether dietary protein serves as a precursor of protoporphyrin

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in the rat, as is indicated by the studies of Schoenheimer and his coworkers (8), by means of certain amino acids containing isotopic nitrogen, and, if so, the proportion of the protein intake allotted to porphyrin synthesis *in vivo*. Fecal protoporphyrin excretion was followed quantitatively in groups of rats fed adequate or low protein diets.

EXPERIMENTAL

Male rats of the Connecticut Agricultural Experiment Station strain weighing 40 to 50 gm. at weaning were used. They were divided litter-wise into two groups and were housed in individual wide mesh screen bottom cages. One group (twelve rats) received an "adequate protein" diet having the following percentage composition: casein¹ 22.5; sucrose 10; white corn dextrin 36.5; hydrogenated cottonseed oil 27; Wesson's salt mixture 4. The other group (twelve rats) was given a low protein diet having the same composition with the following exceptions: casein 3.5; white corn dextrin 55.5. All animals were given the following vitamin supplements: 200 mg. of ryzamin-B² and 200 mg. of liver extract³ daily; 3 drops of halibut liver oil with viosterol twice weekly.

The animals of both groups were weighed and the food consumption was carefully measured weekly. Each day the feces were collected quantitatively on absorbent paper. A 16 week period of observation was used and duplicate protoporphyrin determinations were made biweekly on the feces of each animal.

The following procedure was used for protoporphyrin analysis: The daily feces collection was weighed to 0.01 gm. and was placed immediately in 95 per cent ethyl alcohol and stored in a refrigerator. Pooled weekly samples for each rat were then homogenized in 95 per cent ethyl alcohol in a Waring blender, dried at room temperature, and ground to a fine powder which passed through a 60 mesh sieve. This powder was then dried *in vacuo* at 60°, and duplicate 0.500 gm. aliquots were taken for protoporphyrin determination by a slight modification of the Grinstein and Watson (1) method. The final colored solution was read in a photoelectric colorimeter with a 410 m μ filter. In addition, readings were made with a special 554 m μ filter, a wave-length at which protoporphyrin has a characteristic absorption maximum.⁴ The values obtained at both wave-lengths agreed closely. A calibration curve was constructed with crys-

¹ No. 453, Casein Manufacturing Company, New York.

² No. 2, Burroughs Wellcome and Company, Tuckahoe, New York. Appreciation is expressed to Mr. D. M. Cypher for supplying this material.

³ Liver extract "B," from The Wilson Laboratories, Chicago. Appreciation is expressed to Dr. David Klein for furnishing a generous supply of this extract.

⁴ Dr. R. H. McCoy, University of Pittsburgh; personal communication.

talline protoporphyrin dimethyl ester.⁵ Satisfactory recoveries (average 96.9 per cent) of 30 to 45 γ samples of protoporphyrin added to dried feces were obtained.

Analyses of the two diets (10 gm. samples) employed and of daily portions of the vitamin supplements used demonstrated that the protoporphyrin content of each was only a questionable trace, and hence of no practical significance.

Hemoglobin determinations were made by a photoelectric acid hematin method at regular intervals.

TABLE I

*Fecal Protoporphyrin of Control Rats and of Rats Fed Diet Low in Protein**

Protein diet	Wks.	Fecal protoporphyrin			
		Dried feces	Per rat	Body weight	Protein ingested
		γ per 0.5 gm.	γ per day	γ per 100 gm.	γ per gm.
Adequate	4	42 (29-62)	110 (62-154)	59 (31-93)	62 (32-75)
	6	53 (31-98)	146 (67-224)	56 (24-91)	55 (23-90)
	8	68 (50-102)	182 (136-262)	53 (38-75)	76 (59-129)
	10	73 (33-113)	202 (107-295)	52 (32-81)	78 (38-115)
	12	89 (52-104)	232 (165-344)	54 (39-68)	91 (63-118)
	14	97 (62-122)	241 (179-367)	52 (36-70)	103 (62-149)
	16	86 (52-107)	212 (122-305)	43 (28-55)	92 (53-116)
Low	2	29 (26-35)	19 (16-22)	41 (35-44)	127 (93-164)
	4	31 (16-40)	17 (7-22)	38 (18-48)	143 (86-217)
	6	37 (31-46)	15 (8-19)	30 (19-38)	153 (72-193)
	8	28 (11-55)	13 (5-24)	31 (12-58)	121 (43-232)
	10	23 (2-46)	13 (1-37)	28 (3-67)	123 (11-256)
	12	16 (13-19)	8 (6-11)	17 (12-22)	93 (76-120)
	14	13 (6-19)	7 (2-12)	14 (7-21)	71 (31-111)
	16	12 (11-14)	6 (6-6)	13 (13-13)	94 (92-95)

* The values given are group averages with the minimum and maximum for individual rats in parentheses.

Results

The averaged results for the two groups of rats, together with the range of individual values for fecal protoporphyrin excretion at biweekly intervals, are given in Table I. It is evident that the controls fed the adequate protein diet excreted considerably more protoporphyrin per 0.5 gm. sample, per day, and per 100 gm. of body weight per day than did the

⁵ Sincere appreciation is expressed to Dr. C. J. Watson, University of Minnesota School of Medicine, to Dr. W. J. Turner, Veterans Administration, and Captain John Mason, Army Medical School, for generous samples of protoporphyrin.

rats fed the low protein ration. The difference was evident within 4 weeks after the experiment began and became more pronounced as the study progressed. The rather constant protoporphyrin excretion per 100 gm. of body weight per day observed in the control animals is of interest, as likewise is the steady decrease in this value seen in the low protein rats. This decrease paralleled the development of the chronic anemia usually observed in protein-deficient rats (9), the average value at the end of the 16 week period being 10.7 gm. of hemoglobin per 100 cc. of blood, as compared with 16.8 gm. per 100 cc. for the controls.

The values obtained for fecal protoporphyrin excretion when calculated on the basis of micrograms per gm. of dietary protein consumed show a rather striking contrast between the two groups of rats. The averaged results show clearly that in the first part of the experiment the low protein rats showed a much greater utilization of dietary protein for protoporphyrin formation than did the adequate protein controls. This difference became less pronounced as the experiment progressed, however, perhaps for two reasons. First, there was a general failure in hemopoiesis in the low protein rats, as evidenced by the development of an anemia. Second there was a decreased rate of growth in the control animals, and hence perhaps more protein became available to the organism for porphyrin formation. The data thus suggest that protoporphyrin synthesis, like hemoglobin synthesis (10, 11), has a "high priority call" on available protein in the organism and that porphyrin formation takes precedence over the formation of general body tissue protein when the intake of protein is limited.

DISCUSSION

The foregoing results indicate that if fecal protoporphyrin excretion is a measure of porphyrin synthesis in the organism, as appears to be demonstrated by the existing evidence, dietary protein serves as the ultimate precursor of the porphyrins *in vivo*. This conclusion is in accord with the observations of Schoenheimer and his coworkers (8) previously mentioned.

The question of the identity of the amino acid or acids which serve as precursors for the porphyrin nucleus is one of fundamental and practical importance. Years ago Fischer and others (see (12)) suggested that such substances as proline or tryptophane might serve this purpose. In this connection it is pertinent to note that Shemin and Rittenberg (13) have now demonstrated, using the isotopic nitrogen technique, that glycine is involved in hemin formation, thus in protoporphyrin formation in man. Under the same experimental conditions, no *direct* utilization of either proline, glutamic acid, leucine, or ammonium citrate for porphyrin synthesis could be demonstrated. It is also of interest that acetic acid may be

used in the *in vivo* synthesis of porphyrin, as indicated by the use of isotopic carbon as a tracer (14). Evidently an adequate supply of this important chemical nucleus is thus assured to the organism by synthesis from simple plentiful units. Such a general concept of the importance attached to porphyrin synthesis in the organism is further supported by the data obtained in the present study, demonstrating that protoporphyrin formation, like hemoglobin formation, ranks high in the preferential allotment of protein for anabolic purposes *in vivo*.

SUMMARY

The effect of a diet low in protein (3.5 per cent casein) on fecal protoporphyrin excretion in the rat was followed for a 16 week period.

Porphyrin excretion of the low protein animals was consistently much less than that of control rats when calculated either as micrograms of protoporphyrin per 0.50 gm. of sample, per rat per day, or per 100 gm. of body weight per day, but was significantly greater when expressed as micrograms per gm. of dietary protein ingested.

Protoporphyrin excretion of the low protein rats decreased progressively during the period of observation, while the usual chronic anemia developed.

If fecal protoporphyrin excretion is an index of porphyrin synthesis *in vivo*, as the available evidence indicates, these data demonstrate that dietary protein serves as a precursor of the porphyrin nucleus in the rat and that porphyrin formation, like hemoglobin formation, has a high "priority rating" for available protein in the organism.

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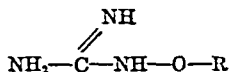
COLORIMETRIC DETERMINATION OF CANAVANINE

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Kitagawa and his colleagues (1-3) reported the isolation of canavanine from jack bean and demonstrated (4, 5) that its structure was $\text{H}_2\text{N}\cdot\text{C}\cdot(\text{NH})\cdot\text{NH}\cdot\text{O}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CHNH}_2\cdot\text{COOH}$. They observed (3, 4) that a solution of sodium nitroprusside that had first been exposed to sunlight reacted with canavanine, specifically, to form a ruby-colored solution. On this finding has been based the development of a simple quantitative method (referred to in an earlier publication (6)) for determining canavanine or, more strictly, compounds having the guanidino grouping



The nature of the reaction involved in the formation of color with canavanine is not known at present. However, conditions for the preparation of the reagent and for the development of color have been standardized so that the reaction can be used for the quantitative determination of canavanine.

Method

Reagents—

1.0 \mathcal{M} phosphate buffer, pH 7.2, is prepared by mixing 0.25 mole (34.5 gm.) of $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$ with 0.75 mole (130.6 gm.) of K_2HPO_4 and adjusting the volume to 1 liter.

Sodium nitroprusside, 2 per cent solution. This is prepared fresh every week and is stored in the dark at 0-4°.

Superoxol (30 per cent H_2O_2).

Potassium carbonate, 20 per cent solution.

Stock solution of canavanine. 2.5 mg. of canavanine¹ per cc. This is conveniently stored frozen on solid CO_2 .

¹ The canavanine used in this investigation was obtained from Squibb's double strength urease as a by-product during the preparation of canavanine-free urease (6). Squibb's double strength urease, prepared from jack bean by acetone precipitation according to the method of Van Slyke and Cullen (7), contains from 6.0 to 8.0 per cent of canavanine. This is easily separated from urease by dialysis. After concentration of the dialysate under reduced pressure the canavanine is precipitated as the flavanate (8, 9) and recrystallized. Addition of excess barium hydroxide results in precipitation of barium flavanate and liberation of the canavanine. Excess barium is removed with CO_2 . Residual flavanic acid is removed from the filtrate with charcoal.

Standard solution of canavanine. 1 cc. of stock solution is diluted to 10 cc. with water.

Preparation of aquoprusside reagent. 0.5 cc. of the 20 per cent carbonate solution and 0.4 cc. of superoxol are mixed with 10 cc. of the 2 per cent sodium nitroprusside solution. After the mixture has stood for 30 minutes, at room temperature (20–25°), it is ready for use. It should be prepared fresh daily. Use of larger amounts of peroxide decreases the color obtained with canavanine.

Procedure

To 1 cc. of each neutral unknown containing 0 to 0.25 mg. of canavanine are added 0.5 cc. of 1.0 M phosphate buffer, pH 7.2, and 0.5 cc. of the aquoprusside reagent. Standards, containing 0.2, 0.5, 0.8, and 1.0 cc. of dilute canavanine stock solution, made up to 1.0 cc. with water, and a reagent blank (1 cc. of water), are treated similarly with phosphate and aquoprusside. The solution in each tube is mixed thoroughly. The tubes are set in a dark closet. A red color develops when canavanine is present. After 2 hours, the color is read in either a visual or photoelectric colorimeter. When a spectrophotometer is used, the instrument is adjusted to a wave-length setting of $\lambda = 520 \text{ m}\mu$ and the blank is set at zero optical density. The authors have used a Coleman Junior spectrophotometer with small cylindrical cuvettes (matched test-tubes 10 mm. in internal diameter). The color development took place in the cuvettes, and the total volume of 2 cc. was adequate. If the colorimeter or cuvettes available require volumes larger than 2 cc., as much as 10 cc. of water may be added to the unknown, standards, and blank *after* the 2 hour period of color development.

Calculation

The optical density of each standard is plotted against the corresponding amount of canavanine. The amount of canavanine in each cc. of unknown is read from the curve so obtained.

Results

The optical density curves of the colored canavanine derivative and of the reagent blank (both read against water) are indicated in Fig. 1. For comparison, the curve given when thiourea replaces canavanine is included.

The extent to which Beer's law is followed is indicated in Fig. 2. Results indicated in Figs. 1 and 2 were obtained with a Coleman Junior spectrophotometer.

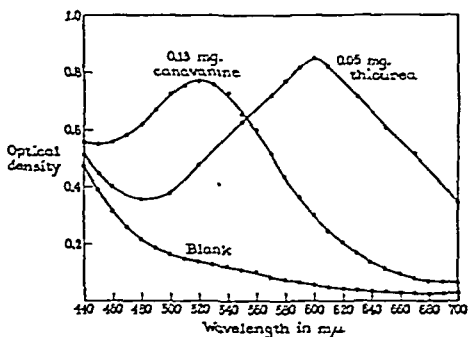


FIG. 1. Optical density curves of the products obtained with canavanine and thiourea, as well as the aquopruesside reagent blank, read against water. Weights refer to the amount of substance in 2 cc. of colored solution. The procedure was conducted as outlined in the method. The readings were taken after 2 hours standing at room temperature.

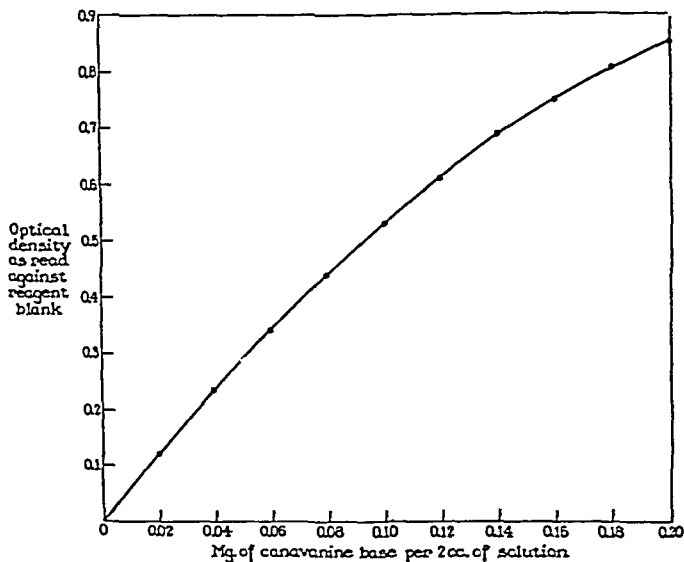


FIG. 2. Curve showing the relation of optical density of the product to the amount of canavanine in 2 cc. of solution. The procedure was conducted as outlined in the method. The readings were taken at a wave-length of 520 mμ against the reagent blank.

For convenience of reference and comparison of results, a constant for the cylindrical cuvettes used is given. These cuvettes give the same reading as do the 1 cm. deep (square cross-section) cuvettes regularly used in a Beckman quartz spectrophotometer. A 0.0125 M solution of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 2 N NH_4OH read at a wave-length of 620 m μ against 2 N NH_4OH had an optical density of 0.73. According to Drabkin and Austin (10) the extinction coefficient of 0.0125 M copper ammonium sulfate under these conditions is 58.

Discussion of Method

The colored product is relatively stable. On standing in the dark an extra 6 hours at room temperature, the optical density at 520 m μ of a standard containing 0.20 mg. of canavanine decreased less than 2 per cent. It had faded 5 per cent after 16 hours. Under similar conditions the color produced with thiourea faded 5 per cent in 2 hours, 20 per cent in 6 hours, and 58 per cent in 16 hours.

Specificity

Although sodium nitroprusside has been used often as a color reagent for the detection of a considerable variety of substances ((11), see p. 184; (12), see p. 397), the only compounds which give an appreciable red color at the pH of 7.2 employed in this procedure are derivatives of hydroxy-guanidine. Canavanine is the first and probably the only derivative yet known to occur naturally. Synthetic derivatives have been studied by Borek and Clarke (13). Although thio compounds, such as thiourea, thiouracil,² and sulfide, react to give a blue color, the appearance and absorption curves are so different from those given by canavanine that interference is unlikely. The color given by thiouracil develops rapidly, then fades, and little remains after 2 hours standing.

The following substances gave no appreciable color with the aquoprusside reagent at pH 7.2: acetone, acetoacetate, arginine, glycoeyamine, creatine, creatinine, guanidine, $(\text{NH}_4)_2\text{SO}_4$. Glutathione and cysteine yield very slight color (gray-purple) with the aquoprusside reagent but the color fades almost completely in 30 minutes.

Notes on Preparation of Aquoprusside Reagent

Treatment of a weakly alkaline solution of nitroprusside with hydrogen peroxide is the simplest means of preparing the aquoprusside compound responsible for the color development in the presence of canavanine.

² There is an obvious similarity of the nitroprusside reagent used here for canavanine and Grote's reagent (14) which is currently employed for measurement of thiouracil (15).

Reagents prepared in this manner have had not only the lowest blanks but also have been the most active. However, it appears desirable to record observations made during the course of the investigation on the nature of the compound responsible for the color development in the presence of canavanine.

Active reagents have been prepared by exposing 2 per cent solutions of sodium nitroprusside to various kinds of light. Solutions placed in an open white porcelain evaporating dish and exposed to sunlight, or, better still, 3 inches from a fluorescent desk light, reach maximum activity in 2 to 4 hours. By use of a quartz mercury vapor lamp, a reagent of satisfactory strength can be obtained by 15 minutes irradiation. As had been observed with sunlight by Qureshi (16), Baudisch (17), and Lal (18), Prussian blue is formed during the prolonged irradiation of nitroprusside solutions. We have noted that this formation of colloidal Prussian blue is accompanied by a drop in pH from an initial value of 7.0 to 5.5. If this fall is prevented by addition of phosphate buffer of pH 7.2, the formation of Prussian blue is markedly inhibited without decreasing appreciably the production of the desired aquopruesside. Formation of Prussian blue can be decreased still further if one uses, instead of strong sources of visible light, the light from a cold quartz mercury vapor tube or from other "cold" mercury vapor lamps such as those employed commercially for sterilization of air. Light, from any source, which has been filtered through Pyrex glass is relatively inactive in producing the desired aquopruesside reagent.

The presence of Prussian blue increases the reagent blank. However, the colloidal sol can be broken readily by shaking 100 cc. of the solution with 1 cc. of alumina gel. Centrifugation yields a brown, active supernatant. No Prussian blue is formed when the aquopruesside is prepared with H_2O_2 .

In connection with the effect of light on nitroprusside solutions, the observations of Baudisch are pertinent (17). He reported that neutral or feebly acid solutions of potassium ferrocyanide, exposed to sunlight or to the light from a carbon arc in the absence of O_2 , yielded ferrous ions and aquopentacyanoferrite ions.¹ In the presence of air and light, the yellow ferrite compound is oxidized to the violet ferrate compound. This in turn is said to react with ferrous ions to form a complex compound having a pale yellow color. Baudisch notes that peroxide compounds having unusual oxidizing power are formed during irradiation in the presence of air. Justin-Mueller (20) claims that sodium nitroprusside ($Na_2Fe(CN)_5NO$) when exposed to air and sunlight yields HCN, $NaNO_2$, and $Na_2Fe_2(CN)_6$. This last compound, in the presence of NaOH, yields Prussian blue. Lal (18),

¹ This oxidized form is known also as sodium pentacyanoaquoferrate (19) or sodium aquopentaferricyanide or sodium cyanoaquopruessate.

however, reports that, on exposure to sunlight, a solution of sodium nitroprusside yields sodium aquopentacyanoferrate⁴ ($\text{Na}_2\text{Fe}(\text{CN})_5\text{H}_2\text{O}$) and part of this is later converted to Prussian blue. Addition of ferrocyanide reduces the ferrate compound to aquopentacyanoferrite ($\text{Na}_3\text{Fe}(\text{CN})_5\text{H}_2\text{O}$). Hofmann (21) has reported that aquopentacyanoferrite is formed when alkaline nitroprusside reacts with H_2O_2 . However, H_2O_2 can oxidize this ferrite compound and the corresponding ferrate compound (18).

We have observed that neither freshly prepared solutions of sodium nitroprusside nor of sodium aquopentacyanoferrate (prepared according to the directions of Hofmann (21)) yield appreciable color with canavanine. Solutions of sodium ammine pentacyanoferrite or of sodium aquopentacyanoferrite, prepared according to Hofmann (21), yield some red color. Further, solutions of sodium aquopentacyanoferrate which have been *partially* converted to the corresponding ferrite compound, either by addition of a small amount of ascorbic acid or of urine, yield more color than the solution of pure ferrite compound.

It is believed, therefore, that maximum color formation with canavanine is dependent upon the presence of both the ferrite and ferrate compounds or on the presence of one or the other, together with a system which converts either one partly to the other.

Interference by Cyanide, SH Groups, and Other Constituents in Biological Preparations

Hill (22) has observed that sodium nitroprusside reacts readily with human or animal tissues or blood to liberate hydrogen cyanide. The nature of this reaction is as yet unknown but the fact that it occurs has been confirmed by the author. Cyanide inhibits the development of color with canavanine in the above reaction, because cyanide converts aquopentacyanoferrite to ferrocyanide. Therefore, one would anticipate that color development would be retarded in solutions containing that component of blood or tissue which frees cyanide from nitroprusside.

We have observed that not only human red cells but also glutathione and cysteine (but not cystine) give rise to a slow liberation of cyanide from nitroprusside. Human blood plasma liberates very little cyanide from nitroprusside.

The decomposition of nitroprusside by blood and by SH groups was demonstrated by use of Conway diffusion units in the following manner. 2 cc. of a 10 per cent solution of sodium nitroprusside were placed in the inner compartment of each unit. Similarly 3 cc. of 0.02 N NaOH were placed in the outer compartment. Then, to the inner compartment, were

⁴ The reduced form is called also sodium pentacyanoaquoferroate (19) or sodium aquopentaferrocyanide.

added 2 cc. of blood cells or of plasma or of cysteine (or of water in the case of the control). The solutions in the inner compartment were mixed with a stirring rod and the units covered and allowed to stand 1 hour. Then 1 cc. of $N H_2SO_4$ was added to the solution in each inner compartment. The units were covered for 2 hours. On addition of 1 cc. of a saturated solution of Ag_2SO_4 in $0.5 N H_2SO_4$ to each of the outer compartments a clear solution was observed in the control. In the case of the units which contained blood or cysteine or glutathione, a curdy white precipitate of silver cyanide was formed. This precipitate dissolved on addition of an equal volume of concentrated HNO_3 .

So far, no method has been found for estimating canavanine in urine or plasma. There is present in urine some unidentified reducing substance which strongly inhibits the development of color with the canavanine reagent. Dialysates of plasma prepared according to the technique of Hamilton and Archibald (23) contain smaller amounts of this inhibitor. Neither urea (5 per cent), $NaCl$ (5 per cent), NH_4^+ , nor creatinine is responsible for the observed effect of urine or plasma dialysates. The inhibitor is a reducing material and is destroyed in part (as is also canavanine) by preliminary oxidation with ceric sulfate or permanganate. Preliminary treatment by addition of oxidizing agents such as H_2O_2 or persulfate, aeration, extraction with organic solvents, or with Lloyd's reagent, or with charcoal, or by precipitation with lead acetate, has failed to make an adequate removal of the interfering substance from a mixture of urine and canavanine. Addition of a mixture of potassium gluconate and potassium persulfate to give an oxidation-reduction potential equal to that of the aquopruesside reagent likewise failed to permit color development when urine is present. Shaking the urine or plasma dialysate for 2 hours with an excess of silver sulfate to remove cysteine or glutathione or related SH compounds decreased the concentration of the inhibitor only slightly.

Attempts to separate canavanine from the interfering substances by adsorption have likewise been unsuccessful. Canavanine is quantitatively adsorbed on Decalso from an aqueous solution. About 70 per cent of the canavanine can be eluted from the permutit by 0.3 per cent $NaCl$ solution and 95 per cent by 1 per cent $NaCl$ solution. However, after a mixture of canavanine and urine (diluted 1:50) had been passed through Decalso and followed by a wash with water, no canavanine could be demonstrated in an eluate made with 1 per cent $NaCl$.

Super Zeo Dur likewise adsorbs canavanine quantitatively from solution and can effect adsorption even in the presence of 3 per cent $NaCl$ solution. Elution of canavanine adsorbed from water or saline solutions can be accomplished with $0.02 N NaOH$. However, such an eluate of a column

through which a mixture of urine and canavanine, followed by water, had passed, failed to give color with the aquoprusside reagent. Neither the Decalso nor the Super Zeo Dur adsorbed from urine substances which on elution interfered with the development of color when aquoprusside and canavanine are present. It would appear, therefore, that either urine prevents adsorption or subsequent elution of canavanine or else something in urine reacts with (and destroys) canavanine.

Before measuring canavanine in any given biological preparations, it is necessary to test the sample for the presence of inhibitor substance. This may be done by comparing the intensity of color obtained on addition of aquoprusside reagent to (a) a solution of purified canavanine and (b) a mixture of the same amount of purified canavanine with an aliquot of the sample to be tested and from which canavanine has been removed by pretreatment with Folin's Decalso. If the intensities are identical (and if a sample treated with Decalso yields no color), no inhibitor is present. If the solution of pure canavanine yields more color, the sample contains inhibitor and the analysis can be conducted as outlined below. Decalso does not appear to remove substances which inhibit development of color.

Application of Method to Analysis of Bean Extracts

20 gm. of Squibb's double strength jack bean urease were made into a paste with 50 cc. of water and dialyzed for 4 hours in cellulose sausage casing against 100 cc. of water at 0° in a manner similar to that employed for the preparation of canavanine-free urease (6). At the end of this time, the concentration of canavanine on either side of the membrane had reached equilibrium. Aliquots of the dialysate were diluted with water and analyzed for canavanine by the procedure outlined above. Canavanine (purified as flavianate), when added to dialysates of some preparations of urease, yielded the expected amount of color with the aquoprusside reagent. Dialysates of a few lots of urease inhibited slightly the development of color as judged by the increment of color resulting from addition of purified canavanine.

Similar dialysates were prepared from jack beans, castor beans, and soy beans. The paste was prepared by soaking the beans in 2.5 parts of water and homogenizing the softened beans in a Waring blender. Jack beans and soy beans yielded dialysates with considerably more inhibitor than did the purified jack bean urease.

Known amounts of purified canavanine were added to dialysates which had been treated with Folin's Decalso to remove canavanine (but not inhibitor). After addition of buffer and aquoprusside reagent as outlined above, standard curves were prepared by plotting the optical densities against the mg. of canavanine added. By comparison with this standard

the amount of canavanine in the untreated dialysate was estimated. The results appear in Table I.

Different aliquots of a solution of purified canavanine were added to 0.5 cc. portions of a dilution of a dialysate of a preparation of urease and the volume was made up to 1 cc. with water. The mixture was treated with phosphate buffer and aquoprusside reagent as outlined in the pro-

TABLE I
Canavanine Contents of Beans and Urease Preparations

Source	Canavanine
	<i>per cent</i>
Squibb's double strength urease prepared from jack beans	
Preparation 1	7.38
	7.40
" 2	6.60
	6.55
" 3	7.10
Jack beans	2.7
Soy "	<0.025
Castor beans	<0.03

TABLE II
Recovery of Canavanine Added to Dialysate of Preparation of Urease

Canavanine added to dilution of dialysate of urease	Total canavanine found	Recovery of added canavanine
<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
None	0.0397	
0.0635	0.1032	99.8
	0.1029	
0.0953	0.1350	100.0
	0.1350	
0.1270	0.1667	100.0
	0.1667	

cedure above. Comparison of the optical densities with the standard curve (prepared as outlined in the preceding paragraph) indicated the values shown in Table II. As might be expected from the manner in which the standards are prepared, the accuracy is about as great as that with which the standard curve is plotted and read.

The author is indebted to Miss E. Stroh for technical assistance.

SUMMARY

1. A quantitative colorimetric method for the determination of 0.01 to 0.25 mg. of canavanine is outlined. A red color develops when canavanine reacts at pH 7.2 with aquoprusside reagent.

2. Thiourea yields with aquoprusside reagent a less stable, blue color. Other organic sulfur compounds yield blue products which are even more unstable.

3. Human red blood cells, glutathione, and cysteine (but neither plasma nor cystine) catalyze the decomposition of sodium nitroprusside to cyanide.

4. Blood filtrates and urine contain unidentified substances which inhibit the development of the color, so that the method in its present form is not applicable to these materials. It appears to be applicable to at least some plant extracts.

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OBSERVATIONS ON SULFUR AMINO ACID DEFICIENCIES IN RATS

CHEMICAL AND MORPHOLOGICAL CHANGES IN THE BLOOD*

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The earliest indication for the need of an organic sulfur complex in the diet came in 1915 from the work of Osborne and Mendel (1), who showed that when the proportion of casein in the diet of the rat was reduced from 18 to 9 per cent the resulting impairment of growth could be corrected by a supplement of cystine equal to 3 per cent of the casein. This finding was confirmed in many laboratories and the rôle of cystine as a growth essential seemed definitely established. However, when in 1932 Jackson and Block (2) observed that methionine is also capable of supplementing a low casein ration, the growth essentiality of cystine was placed in doubt. Later Womack, Kemmerer, and Rose (3) using a synthetic mixture of nineteen amino acids as the protein moiety of an experimental diet concluded (a) that cystine is not a dietary essential for growth of the immature rat, (b) that its presence does not improve the quality of a ration which is adequate in methionine, and (c) that in the absence of methionine the administration of cystine fails to promote growth. These observations implied an irreversible conversion of methionine into cystine which has since been confirmed by the radioactive isotope investigations of Tarver and Schmidt (4). More recent experiments by Womack and Rose (5) have shown that cystine is capable of stimulating growth only when methionine is present in suboptimal amounts and that only 16 per cent of the methionine requirements can be met by cystine.

In apparent contradiction to these growth studies, an extensive study of the relationship of dietary cystine and methionine to the blood proteins led Whipple and coworkers (6) to conclude that methionine is not an efficient substitute for cystine in the regeneration of plasma proteins of experimentally hypoproteinemic dogs. These observations suggested that under certain conditions the rate of biological conversion of methionine is not

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adequate for the normal biosynthesis of plasma proteins, and prompted us to determine the effect of cystine- and methionine-deficient diets on these cystine-rich tissues in animals not subjected to plasmapheresis. These experiments were performed and the results indicate that the blood protein levels of immature and adult rats were not affected by the cystine-deficient diet, whereas they were markedly reduced within 100 days by a methionine-deficient diet. In view of the known high cystine content of blood albumin and globulin, these findings can hardly be interpreted as disqualifying cystine as an amino acid essential in the formation of these vital proteins, but seem rather to furnish additional evidence for the conversion of methionine into required cystine in the animal organism. These observations would seem to complement rather than confute those of Whipple which in effect indicate that greater nutritional efficiency can be gained by feeding the animal such amino acids as are specifically required for specific proteins than can be attained from the feeding of biochemical counterparts.

EXPERIMENTAL

Animals—The observations reported here were made on immature and adult rats derived from a hybrid colony of albino and hooded Norwegian rats. The food intake of the control animals was restricted to that of the animals on the deficient diets. The animals were kept in individual cages which were not designed to prevent coprophagy.

Preparation of Diets—The composition of the diets employed in this study is shown in Table I. The protein moiety of the cystine-deficient diet (Cy) was obtained by the addition of 1.5 per cent of *l*(-)-tryptophane to a neutralized acid hydrolysate of casein previously filtered through norit A. This process has been found to reduce the cystine content of the casein hydrolysates to 0.05 ± 0.02 per cent. The control for this diet (CCy) was derived by supplementation of the protein component of Diet Cy with 1.0 per cent of cystine. The protein fraction of the methionine- and cystine-deficient diet (SF) was prepared by tryptophane reinforcement of a hydrogen peroxide-oxidized acid hydrolysate of casein (7) which contained 0.20 per cent of methionine and 0.17 per cent of cystine by analysis (8, 9). The control diet (CSF) was obtained by the addition of 3.0 per cent of *dl*-methionine and 1.0 per cent of *l*(-)-cystine of the protein fraction to Diet SF. In order to maintain the protein level of the diets constant, the amount of hydrolysate added to the diets was reduced proportionately to the amount of amino acid supplementation. Owing to uncertainties regarding the B complex vitamins, brewers' yeast was used instead of a mixture of the synthetically available components of this vitamin group. From analysis in the literature it appears that the 0.4

gm. of yeast present in each 10 gm. of these diets provided the animals with an additional 1.2 mg. of cystine (10) and 8.0 mg. of methionine (11). This amount of methionine is apparently not sufficient to cover the needs of the immature or adult rat.

Blood Analysis—The blood samples (1.0 to 2.5 cc.) were all obtained by heart puncture and collected over lithium oxalate in 5 cc. centrifuge tubes. After removal of samples for colorimetric hemoglobin determination and cell counts, the remainder was centrifuged and the non-protein

TABLE I
Composition of Diets

	Diet SF	Diet CSF	Diet Cy	Diet CCy
	gm	gm.	gms.	gms.
Acid-hydrolyzed casein*	0	0	15.7	15.5
Oxidized casein hydrolysate* ..	15.7	15.1	0	0
Brewers' yeast†	4.0	4.0	4.0	4.0
Sucrose	14.1	14.1	14.1	14.1
Starch	39.3	39.3	39.3	39.3
Agar	2.0	2.0	2.0	2.0
Crisco	18.0	18.0	18.0	18.0
Cod liver oil substitute‡	4.7	4.7	4.7	4.7
Salt mixture‡	2.0	2.0	2.0	2.0
l(-)-Tryptophane	0.23	0.23	0.23	0.23
l(-)-Cystine	0	0.17	0	0.17
dl-Methionine... ..	0	0.47	0	0
Total ..	100.0	100.0	100.0	100.0

* N \times 6.25 = gm. of protein.

† Mead Johnson and Company.

‡ The salt mixture employed had the following composition (measured in gm.): NaCl 18.9, CaHPO₄, anhydrous, 25.0, MgSO₄, anhydrous, 6.86, KHCO₃, 44.4, KCl 2.88, Fe⁺⁺⁺ citrate, U. S. P., 2.21, CuSO₄, anhydrous, 0.24, MnSO₄, anhydrous, 0.15, KI 0.015, NaF 0.03.

nitrogen and plasma protein estimations performed as previously reported by us (12).

Results

The nutritional adequacy of the diets employed in these studies was bioassayed in the immature rat. It will be observed that as might be expected the growth value of Diet Cy which contains 16 per cent of protein is not improved appreciably by cystine supplementation (Fig. 1). It is evident from the data presented in Fig. 2 that Diet SF, constructed from tryptophane-supplemented peroxide-oxidized casein hydrolysate, is

clearly deficient in methionine, since nutritional adequacy is restored on supplementation with 3 per cent of *dl*-methionine, which provides the animals with 26.0 mg. of *dl*-methionine per 5 gm. of the daily ration. Since Diets SF and Cy are of similar cystine content, and since Diet Cy supports growth in the immature rat (Fig. 1), the growth failure of Diet SF must be due to a lack of methionine rather than cystine.

In appraising the results of the blood studies, attention is called to two previous findings (*a*) that, while a symmetrical reduction in food intake (a sequel of paired feeding experiments) results in poor or negative weight

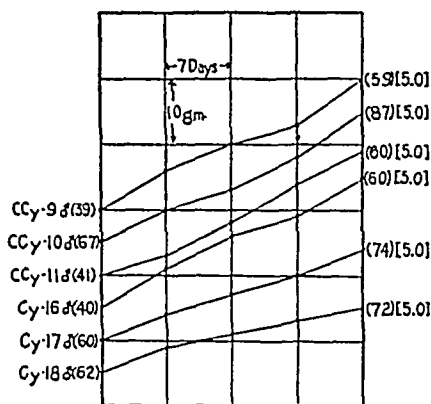


FIG. 1

FIG. 1. The effect of a cystine-deficient diet (denoted by Cy in the rat number) and a cystine-supplemented cystine-deficient diet (CCy) on the growth of the immature rat. The figures in parentheses denote animal weights in gm.; those in brackets, the average daily food intake.

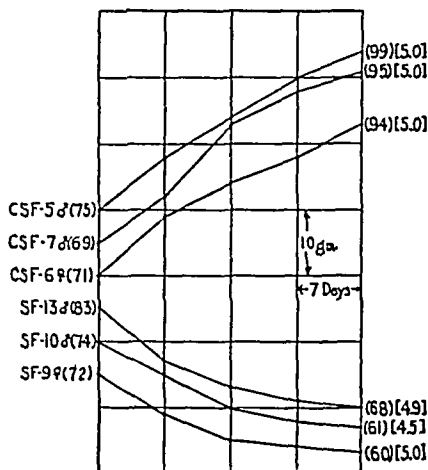


FIG. 2

FIG. 2. Growth effect of methionine (denoted by CSF in the rat number; SF indicates the control diet) on immature rats. The figures in parentheses denote body weights of the animals in gm.; those in brackets, the average daily food intake.

gains of the control animals, it does not affect the hemoglobin or plasma protein levels of the blood, and (*b*) that the plasma protein and non-protein nitrogen levels tend to be higher in the adult rats than in the immature animals (12). Thus, plasma protein values were found to average 6.4 gm. per cent in the normal adult animals in contrast to 5.2 gm. per cent in the immature ones. A comparison of typical values secured for the animals on the cystine-deficient and cystine control diet reveals that a prolonged cystine deficiency failed to induce a reduction of blood protein and cell count levels below those found in the control animals (Table II).

In contrast to these findings, the animals maintained on the methionine-

TABLE II

Blood Studies on Rats Maintained on Cystine Control and Cystine-Deficient Diets

	Rat CCy-1 ♂	Rat CCy-2 ♂	Rat CCy-5 ♀	Rat CCy-7 ♀	Rat CCy-8 ♂	Rat CCy-4 ♀	Rat CCy-3 ♂	Rat Cy-1 ♂	Rat Cy-6 ♂	Rat Cy-9 ♀	Rat Cy-7 ♂	Rat Cy-3 ♀	Rat Cy-10 ♀	Rat Cy-4 ♂	Rat Cy-5 ♀
Initial body weight, gm.	74	60	40	45	41	254	306	82	30	40	44	37	45	320	200
Days on diet	120	170	160	134	134	100	205	180	134	134	134	134	150	186	186
Total body weight change, gm.	+40	+30	+33	+30	+34	-60	-31	+41	+36	+33	+28	+37	+20	-30	-20
Average daily food intake, gm.	4.05	4.05	4.00	4.86	4.98	10	10	4.02	3.05	3.01	3.93	3.92	4.02	10	10
Total plasma protein, gm. %	5.55	5.82	5.50	5.75	5.40	6.88	6.35	5.87	5.67	5.40	5.74	5.90	6.05	5.90	6.01
Albumin, gm. %	3.70	3.70	4.15	3.90	3.70	3.40	3.20	3.98	4.05	4.10	3.82	3.90	4.20	3.22	
Globulin, gm. %	1.85	2.06	1.35	1.85	2.00	3.30	3.09	1.90	1.62	1.30	1.92	2.00	3.85	2.68	
Albumin-globulin ratio	2.00	1.83	3.08	2.10	1.85	1.03	1.00	1.05	2.50	3.15	1.90	1.95	1.00	1.20	
Non-protein N, mg. %	53	53	50	32	30		31	50	61	50	32	45	33	55	50
Hemoglobin, gm. %	15.5	16.0	16.2	14.4	10.0	15.4	15.4			13.8	14.0	14.8	15.9	15.3	14.1
Red blood cells, millions per c.mm.		7.03	9.69	7.72	5.00						6.02	7.60	7.82		
White blood cells per c.mm.		6000	6800	8450	7400			6900				6700	6400		

TABLE III
Blood Studies on Rats Maintained on Control Sulfur-Free and on Sulfur-Free Diets

	Rat CSF-1 σ	Rat CSF-2 ϕ	Rat CSF-4 σ	Rat CSF-10 ϕ	Rat CSF-12 σ	Rat CSF-9 σ	Rat SF-7 ϕ	Rat SF-8 σ	Rat SF-4 σ	Rat SF-5 ϕ	Rat SF-6 ϕ	Rat SF-14 σ	Rat SF-15 σ
Initial body weight, gm.	105	99	128	240	295	335	91	113	137	106	250	303	325
Days on diet	99	136	150	125	120	95	99	99	150	126	130	120	90
Total body weight change, gm.	+38	+39	+27	-30	-63	-95	-21	-32	-36	-16	-103	-160	-150
Average daily food intake, gm.	5.0	5.0	5.0	10.0	10.0	10.0	4.78	4.88	5.39	4.95	8.4	8.9	8.3
Total plasma protein, gm. %	5.74	5.28	5.45	6.25	6.10	6.49	4.52	4.60	4.92	4.67	4.78	4.45	4.39
Albumin, gm. %	4.18	3.69	3.80	4.16	4.37	4.39	2.90	3.42	3.35	3.19	3.00	2.80	2.96
Globulin, gm. %	1.56	1.59	1.65	2.09	1.73	2.10	1.62	1.18	1.57	1.48	1.78	1.65	1.43
Albumin-globulin ratio	2.68	2.32	2.30	1.99	2.50	2.09	1.78	2.90	2.13	2.15	1.69	1.70	2.07
Non-protein N, mg. %	83	130	62	64	42	68	33	72	54	57	55	45	53
Hemoglobin, gm. %	15.4	16.2	16.9	15.4	15.7	15.1	11.3	12.5	11.8	9.5	11.8	12.1	11.6
Red blood cells, millions per c.mm.	8.18	7.90		9.42	9.49		8.29	8.43				9.18	8.75
White blood cells per c.mm.	6350	7400		7350	8500		7950	4600				5900	7300

and cystine-deficient diet (Table III) showed a reduction in total plasma proteins which became consistent and striking after 100 days or more of this regimen, which provides a total of 26.7 mg. of cystine per 10 gm. of daily ration. The loss arises principally from a decrease in the albumin fraction, which is not of such magnitude as to cause conspicuous changes in the albumin-globulin ratios. Furthermore since the marked drop in hemoglobin values caused by the methionine-deficient diet is not accompanied by a fall in red blood cell count, the anemia so induced, like that of the tryptophane and other nutritional deficiencies, is hypochromic in character.

These experiments indicate that in the presence of methionine, cystine is not a dietary essential for the maintenance of normal blood protein levels in the adult and immature rat, but should not be construed as attributing a specific antianemic function to methionine. It is also to be noted that hypoproteinemia and anemia induced by the methionine deficiency in the rat are not as severe as that caused by a tryptophane deficiency of comparable duration.

Comments

The need for cystine in the biosynthesis of plasma proteins is predicated by the relatively high cystine content of the rat plasma proteins. Calculations from the diets employed in these experiments indicate that in the absence of methionine normal blood protein formation cannot be achieved by immature and adult rats on a respective 13.5 and 27 mg. daily intake of cystine. It follows therefore that, in the absence of an adequate supply of dietary cystine, the cystine required for vital proteins must be derived from the metabolic conversion of methionine to cystine. Our experiments, consequently, do not disqualify cystine as an amino acid essential in the formation of plasma proteins, but rather constitute additional evidence of the transformation of methionine into the needed cystine. Moreover, the failure of the cystine-deficient diet to lower the plasma proteins of the rats would seem to support rather than disprove the view of Whipple and his associates that cystine qualifies as a key amino acid in plasma protein regeneration of experimentally hypoproteinemic dogs. Thus, our data demonstrate that cystine required for plasma protein formation can be derived, when necessary, from dietary methionine, while Whipple's observations suggest that greater nutritional efficiency is gained when preformed cystine is available to the depleted animal.

In view of the low cystine content of hemoglobins, it seems reasonable to assume that the anemia induced by the methionine- and cystine-deficient diet results from the dietary lack of methionine rather than cystine.

Attention is called to our report that two adult males maintained on a

diet deficient in both methionine and cystine for 36 days did not reveal any significant changes in blood protein (13). The divergence of our findings in experiments on the rat and human undoubtedly arises from the relatively short duration of the human study.

In this and previous experiments, we have noted that the growth of immature rats on diets prepared with cystine- and tryptophane-supplemented acid digests of casein (14) or mixtures of the crystalline amino acids (15) is suboptimal when compared to the growth of immature animals maintained on diets made up of amigen or whole casein. Inasmuch as the blood protein levels of immature and adult rats maintained on our control Diets CCy and CSF and those on Diet CTH (12) of a previous report fall well within the range of animals maintained on a stock or amigen diet, it can be reasonably assumed that the growth factor deficiency of our diets failed to affect appreciably the production of blood tissue in rats within the period of the experiments.

SUMMARY

It has been found that a cystine-deficient diet does not affect the blood protein levels of the immature or adult rat, whereas hypoproteinemia and anemia are induced in these animals by a methionine- and cystine-deficient diet. These findings indicate that in the dietary absence of cystine the cystine required for the formation of blood albumins and globulins can be derived from methionine. It also appears that the dietary lack of factors, which causes suboptimal growth of the immature rats, does not influence the blood protein levels of these animals.

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DIETARY PROTEIN AND THE VITAMIN B₆ CONTENT OF MOUSE TISSUE*

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It is now generally agreed that vitamin B₆ is involved in the metabolism of amino acids. Certain derivatives of the vitamin function as a coenzyme in the decarboxylation of tyrosine (1, 2), lysine (3), arginine (3), and glutamic acid (3), and also in transamination reactions (4, 5). Vitamin B₆ tends to minimize the toxicity observed when *D*-serine is injected or fed by stomach tube (6, 7). Several species of animals excrete xanthurenic acid, when the vitamin B₆ content of the diet is inadequate; xanthurenic acid is an abnormal metabolic derivative of tryptophane (8, 9). Rats deprived of vitamin B₆ develop dermatitis more readily on diets containing 30 to 60 per cent of casein than when 15 to 18 per cent is fed (10, 11). Mice deprived of vitamin B₆ have a higher mortality rate on high protein diets than when the protein content of the diet is moderate; the amount of vitamin required for a given growth response rises as the consumption of protein is increased (9). These observations raised the question whether the consumption of protein exerts an adverse effect upon the concentration of vitamin B₆ within the animal body.

EXPERIMENTAL

Methods

Young rats or mice were fed pyridoxine-deficient diets that contained 10 or 50 per cent of casein, and, after suitable intervals of time, the animals were killed and the tissues analyzed microbiologically for vitamin B₆ (*Streptococcus carlsbergensis* assay 12). In other series graded amounts of pyridoxine were added to the two basal diets fed. Other dietary variations included changes in the kind of protein or in the other vitamins of the B complex (see below).

All animals were kept in screened cages, the rats singly, the mice in groups of three to four. The rats usually weighed 40 to 50 gm. at the beginning of the experiment; the mice weighed 9 to 14 gm. They were

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fed the diets listed in Table I. Periodically the mice were placed for 24 hours in metabolism cages fitted with an outside feeder and the urine was collected under toluene. At first the vitamin B₆ was determined both in diluted unhydrolyzed urine and in aliquots that had been autoclaved for 1 hour in 0.1 N HCl. However, the same values were obtained with both procedures, and hence subsequent determinations were made on unhydrolyzed urine only.

The tissues were prepared for analysis as follows: the animal was decapitated and allowed to bleed freely. The livers, kidneys, and muscle from the hind legs were removed, and the smaller organs, or 1 gm. aliquots

TABLE I
Composition of Rations Fed

	Mice	Rats
	<i>per cent</i>	<i>per cent</i>
Casein (alcohol-extracted).....	10 or 50	10 or 50
Corn oil*.....	5	5
Wesson's salts.....	4	4
Cerelose† to.....	100	100
	<i>mg. per 100 gm. diet</i>	<i>mg. per 100 gm. diet</i>
Pyridoxine hydrochloride.....	0-1.0	0-0.25
Thiamine chloride.....	0.3	0.25
Calcium pantothenate.....	2.0	2.0
Riboflavin.....	0.6	0.3
Nicotinic acid.....	0.5	0.25
Choline.....	50	100
Inositol.....	50	100
p-Aminobenzoic acid.....	30	30
Cystine.....	100	100

* 1 volume of halibut liver oil was added to 999 volumes of corn oil. Each rat also received 2 drops of halibut liver oil at the start of the experiment.

† Glucose monohydrate.

of the larger ones, were weighed immediately and homogenized in a Waring blender for 2 to 3 minutes with 25 ml. of 0.1 N HCl. The samples were then diluted to approximately 180 ml. with 0.1 N HCl and autoclaved for 1 hour at 15 pounds pressure. Essentially the same values were obtained with 0.055 N HCl as with 0.1 N HCl. The samples were cooled and the pH adjusted to 5.2, chilled, and the precipitate allowed to coagulate. Filtered aliquots were then added to suitably prepared medium inoculated with *Streptococcus carlsbergensis* according to Atkin *et al.* (12) and the turbidity measured after incubating at 30° for 16 hours. A standard curve was made within the range of 0 to 50 millimicrograms of pyridoxine per

flask with each series of determinations. All manipulations were carried out in subdued light.

The results were expressed as micrograms of pyridoxine per unit weight of sample, although it is recognized that pyridoxal and pyridoxamine are also present in tissues (13). However, the organism employed in the assay responds to all three forms approximately as higher animals do (14), and hence the assay is a measure of vitamin B₆ taken collectively.

Results

Effect of Casein on Depletion of Hepatic Vitamin B₆—Weanling mice were found to contain 4.4 γ of vitamin B₆ per liver or 9.8 γ per gm. of liver tissue. This corresponds fairly well with the amounts found in the livers of other species (12, 15). When vitamin B₆ was omitted from the diet, the amount in the liver decreased, whether the percentage of casein in the

TABLE II
Depletion of Vitamin B₆ from Livers of Mice Fed 10 or 50 Per Cent Casein

		Wks. on experiment						
		0	2	3	5	7	9	12
10% casein	No. of mice	3	3	4	4	4	5	3
	Average weight, gm.	9.5	12.8	13.0	12.0	11.3	10.8	10.4
	Vitamin B ₆ per liver, γ	3.6	2.9	2.9	3.2	1.4	1.1	0.87
	" " " gm. liver, γ	8.5	4.3	3.7	4.2	2.2	1.5	1.25
50% casein	No. of mice	3	3	2	All dead			
	Average weight, gm.	8.7	8.1	7.8				
	Vitamin B ₆ per liver, γ	3.6	1.5	0.85				
	" " " gm. liver, γ	8.5	1.9	0.97				

diet was high or low (Table II). The rate of decrease was much greater on the higher level of protein. After 3 weeks on 50 per cent of casein, mice contained only 0.85 γ of vitamin B₆ per liver (0.97 γ per gm.), whereas mice on 10 per cent of casein still contained 2.9 γ per liver (3.7 γ per gm.). On the low protein diet a period of 12 weeks was required before the concentration of hepatic vitamin B₆ decreased to the low level reached in only 3 weeks on 50 per cent of casein (Table II).

In general the changes in the concentration of vitamin B₆ per gm. of liver tissue paralleled the changes per liver, although some irregularities were evident. Thus during the first 2 weeks on 10 per cent of casein the mice gained in weight and the livers also increased in size, while the total amount of vitamin B₆ present per liver decreased by 19 per cent (Table II). The concentrations per gm. of liver decreased by 50 per cent during this interval. On the 50 per cent casein diet, also, the percentage decreases

per gm. of liver were somewhat greater than those per liver, 58 and 77 per cent respectively at 2 weeks. Changes in the other organs were roughly similar to those in the liver.

The amounts of hepatic vitamin B₆ retained when the deficient diets were fed depended among other things upon the size of the mice at the beginning of the depletion period (Table III). This effect was not very great on the diet containing 10 per cent of casein, but on 50 per cent of casein the amounts of vitamin B₆ in the liver ranged from averages of 1.3 γ to 3.9 γ , depending upon whether the mice weighed 8 or 14 gm. at the beginning of the depletion period. Mice of intermediate sizes contained intermediate amounts of hepatic vitamin B₆ (Table III). The heavier mice on 50 per cent of casein contained as much vitamin as those on the low protein diet (ca. 4 γ per gm.). Higher levels of casein increased the rate of depletion

TABLE III

*Effect of Protein on Depletion of Hepatic Vitamin B₆ in Mice of Different Sizes
(Depletion Period, 3 Weeks)*

Initial weight of mice	10 per cent casein diet		50 per cent casein diet	
	Vitamin B ₆ per liver	Vitamin B ₆ per gm. liver	Vitamin B ₆ per liver	Vitamin B ₆ per gm. liver
gm.	γ	γ	γ	γ
8-9	2.6 (4)	4.1	1.3 (7)	1.8
9-10	2.3 (5)	3.9	1.4 (5)	1.5
10-11	2.4 (7)	3.4	1.8 (2)	1.7
11-12	3.3 (4)	3.9	2.2 (4)	3.3
12-14	4.2 (5)	4.7	2.7 (4)	3.7
14-17	4.1 (4)	4.1	3.9 (6)	4.3

The numbers of mice used for each determination are indicated in parentheses.

of vitamin B₆ from adult mice, although more time was required for the effect to become evident. After 5 to 9 weeks of depletion livers of mice fed 10 per cent of casein contained 4.0 γ of vitamin B₆ per gm. (2.3 to 5.2), as compared to 2.4 γ (1.6 to 3.2) on 50 per cent of casein. Per liver the figures were 4.3 γ (3.7 to 4.9) and 2.9 γ (1.8 to 2.7) respectively.

The faster rate of depletion of vitamin B₆ on the high protein diet did not appear to be due to an increased loss of vitamin in the urine. Similar amounts of vitamin B₆ were found in the urine from the low and high casein groups: 0.21 and 0.23 γ respectively per adult mouse per day after 3 weeks, and 0.44 and 0.43 γ after 7 weeks. During the 1st week of depletion weanling mice excreted 0.21 and 0.18 γ of vitamin B₆ daily on the two diets respectively, while the amounts during the 3rd week were 0.12 and 0.15 γ . Nor did the lower amounts of vitamin B₆ in the liver

appear to be due to a reduction in food intake on the higher level of casein. Weanling mice were fed the 10 per cent casein diet in amounts calorically equivalent to those consumed by a control group on 50 per cent of casein, while a third group received the 10 per cent casein diet *ad libitum*. During a 3 week period the consumption of food averaged 2.5 gm. per day per 10 gm. of mouse on the 10 per cent casein diet, and only 1.44 gm. per day on the 50 per cent casein diet.

The livers of eight mice fed restricted amounts of the 10 per cent casein diet contained as much vitamin B₆ as those fed the diet *ad libitum*, 2.5 and 2.4 γ per liver respectively. However, the partially fasted mice had the smaller livers, and hence the concentration of vitamin per gm. of tissue was actually increased 4.5 and 3.4 γ per gm. respectively. Eight

TABLE IV
Storage of Vitamin B₆ in Livers of Mice Fed Graded Amounts of Pyridoxine in High and Low Protein Diets

Pyridoxine per 100 gm. diet	10 per cent casein				50 per cent casein			
	No. of mice	Average growth per wk.	Vitamin B ₆		No. of mice	Average growth per wk.	Vitamin B ₆	
			Per liver	Per gm. liver			Per liver	Per gm. liver
mg.		mm.	γ	γ		mm.	γ	γ
0	23	0.8	2.7	3.7	18	-0.3	1.9	2.5
0.05	2	2.3	6.2	5.8				
0.10	10	2.3	7.7	6.5	9	1.3	6.6	5.8
0.25	13	2.2	8.6	10.1	13	2.2	10.7	10.0
0.50	7	2.1	9.5	9.1	5	2.6	14.7	12.5
1.0	11	2.2	9.8	9.6	11	2.6	14.3	11.1

mice of this series on 50 per cent of casein averaged 2.3 γ of vitamin B₆ per gm. of liver tissue.

Effect of Casein upon Storage of Vitamin B₆.—Young mice 8 to 11 gm. in weight were fed either the high or the low protein diet with amounts of pyridoxine ranging from 0 to 1.0 mg. per 100 gm. of diet (Table IV). The rations were fed for 3 to 4 weeks and the hepatic vitamin B₆ determined. As observed previously, the mice deprived of pyridoxine lost weight on 50 per cent of casein, while those on 10 per cent of the protein gained weight slightly. Optimal growth, within the limits permitted by the diet, was observed on the low protein diet when 0.05 mg. of pyridoxine was added per 100 gm. of ration. On the high protein diet, however, 5 times as much, *viz.*, 0.25 mg. of pyridoxine, were required per 100 gm. of ration for a similar growth response (Table IV). This confirms a previous observation that a high protein diet increases the requirement of pyridoxine for the growth of mice (9).

A parallel observation was that the hepatic storage of the vitamin on low levels of pyridoxine intake was somewhat lower on 50 per cent of casein than on 10 per cent of casein; *e.g.*, 5.8 as compared to 6.5 γ of vitamin B₆ per gm. of liver at an intake of 0.1 mg. per 100 gm. of diet (Table IV). At higher levels of pyridoxine intake, however, 0.5 to 1.0 mg. per 100 gm., the high protein diet actually appeared to promote the storage of the vitamin. Livers of mice on the low protein diet contained 9.1 to 9.6 γ per gm., as compared to 11.1 to 12.5 γ per gm. on 50 per cent of casein.

Thus dietary protein appeared to exert opposite effects upon the storage of hepatic vitamin B₆, depending upon the level of vitamin in the diet. At low levels of vitamin intake high protein minimized vitamin storage. This may well be a reflection of a greater need for vitamin B₆ when large

TABLE V

Effect of Certain Proteins and Amino Acids on Depletion of Vitamin B₆ from Mouse Livers (3 Weeks)

Group No.		No. of mice	Gain per wk.	Vitamin B ₆	
				Per liver	Per gm. liver
	Initial stores	8	gm.	γ	γ
1	10% casein	7	0.7	4.4	9.8
2	20% "	8	0.7	2.8	3.2
3	20% fibrin	8	0.7	2.5	3.3
4	20% fibrin	7	0.9	3.5	3.6
5	20% egg albumin	4	1.3	5.2	5.9
6	20% lactalbumin	4	1.0	3.4	3.3
7	50% casein	6	-0.4	2.1	2.4
8	10% " + 0.96% <i>dl</i> -tryptophane	11	0.6	2.7	3.7
9	10% casein + amino acids*	4	0.8	3.3	5.3

* Amino acids added as per cent of diet: *l*(+)-glutamic acid 9.12, *l*-asparagine 2.52, *l*-tyrosine 2.56, *dl*-alanine 1.20, *dl*-phenylalanine 0.40, glycine 0.22.

amounts of protein are being metabolized. On the other hand protein tended to promote the storage of vitamin B₆ when the intake of the vitamin was high, possibly by increasing the concentration of those substances to which the vitamin is attached in the liver. In this connection it is of interest that the hepatic storage of riboflavin is also much higher on high protein diets than when the intake of protein is low (16).

Effect of Other Proteins and Amino Acids—The marked effects of casein upon the storage of vitamin B₆ in the liver raised the question whether other proteins produced a similar response. Tryptophane was studied in this connection because its metabolism is abnormal in vitamin B₆ deficiency. Accordingly young mice were fed for 3 to 4 weeks on diets deficient in vitamin B₆ and containing the proteins or amino acids listed in Table V.

20 per cent of fibrin was fed because this amount contains as much tryptophane as 50 per cent of casein (17). For comparison casein, egg albumin, and lactalbumin were also fed at the 20 per cent level. The *dl*-tryptophane in Group 7, Table V, was calculated to supply as much of the *l* isomer as the "extra" 40 per cent of casein in the 50 per cent casein diet (Group 6). The final group (Group 8) contained 10 per cent of casein and 16 per cent of a mixture of *dl*-alanine, *dl*-phenylalanine, *l*-tyrosine, *l*-asparagine, and *l*-glutamic acid. These amounts, also, were equivalent to the *l* acids present in 40 per cent of casein.

The mice on 50 per cent of casein (Group 6) lost an average of 0.4 gm. per week and their livers contained less vitamin B₆ than those of Group 1, which received 10 per cent of casein. The latter mice gained 0.7 gm. per week. All other groups grew at least as well as those on 10 per cent of casein, and contained as much vitamin in the liver. On many of the diets the original amount of vitamin B₆ in the liver, 4.4 γ , did not decrease much during the 3 weeks of the experiment, *i.e.*, to 2.7 to 3.5 γ per liver. Per gm. of liver, however, there was a marked decrease in all cases, presumably because the mice gained in weight and a limited amount of vitamin, although conserved rather efficiently, was distributed in more liver tissue. The mice on egg albumin and lactalbumin (Table V, Groups 4 and 5) grew best of all, and their livers were somewhat higher in vitamin B₆ than those in the other groups. However, these proteins contained 0.017 and 0.013 mg. of vitamin B₆ per 100 gm. of ration, and hence the favorable effects observed are presumably without significance.

It was evident, however, that tryptophane was not the main factor responsible for the depletion of the vitamin from the liver of mice on high protein diets. When *dl*-tryptophane was added to a 10 per cent casein diet, growth and storage were very similar to those when the tryptophane was omitted (Table V, Groups 7 and 1). On fibrin (Group 3), which is high in tryptophane, growth and storage were actually improved somewhat. The amino acid mixture (Group 8) did not affect growth or the storage of the vitamin, although the livers decreased in size and hence the concentration of vitamin B₆ per gm. of liver appeared to be increased.

Fatty Livers—Many of the mice on the diets low in vitamin B₆ developed pale, friable, fatty livers. This condition appeared much more rapidly on the high casein diet than on that containing only 10 per cent of casein (Table VI) and, in general, seemed to parallel the other evidences of a severe vitamin B₆ deficiency such as loss in weight and a low concentration of vitamin in the tissues. The addition of tryptophane to the diet either as the *dl* acid or as fibrin tended to hasten fatty liver formation.

Preliminary analyses indicated that roughly 15 per cent of the fresh weight of such livers was extractable with ether. When a fatty liver was homogenized in acid for vitamin B₆ analysis, an extremely cloudy and stable

emulsion resulted, which, however, was broken after autoclaving and neutralization to pH 5.2. The filtered aliquots added to the culture tubes were clear. Nevertheless, the question arose whether the presence of fat might have interfered with the assay for vitamin B₆. Accordingly four large livers from normal mice were minced and divided into two portions each. The first was analyzed directly; the second (ca. 0.6 gm.) was added to 1 ml. of corn oil prior to homogenization and extraction. In no case did the presence of this added fat alter the results of the analysis for vitamin B₆, and hence the low levels of the vitamin found in the livers of mice fed 50 per cent of casein were regarded as real.

TABLE VI
Fatty Livers in Mice Fed Diets Deficient in Vitamin B₆

	Wks. on experiment	No. of mice	No. with fatty livers*	Per cent
10% casein.....	3-4	31	0	0
10% ".....	7	4	1	25
10% ".....	8	5	2	40
10% ".....	12	4	4	100
50% ".....	3-4	30	16	53
10% " + dl-tryptophane.....	3-4	11	3	27
20% fibrin.....	3-4	7	2	29
20% egg albumin.....	3-4	4	4	100
20% lactalbumin.....	3-4	4	0	0
20% casein.....	3-4	12	0	0
20% " + biotin† + <i>Lactobacillus casei</i> factor†.....	3-4	4	0	0
20% casein + biotin† + <i>L. casei</i> factor† + 0.5% succinylsulfathiazole.....	3-4	3	0	0

* Based only on the gross appearance of the livers.

† 5 γ per gm.

Vitamin B₆ in the Rat—Weanling rats were deprived of vitamin B₆ for 10 weeks on diets containing 10 or 50 per cent of casein, and the tissues analyzed as before. Control animals received 2.5 γ of pyridoxine per gm. of diet. In contrast to the results with mice, the high protein diet did not hasten the depletion of vitamin B₆ from the tissues of the rats (Table VII), nor was the storage of the vitamin affected at a comparatively high level of pyridoxine intake. The rats receiving pyridoxine and 50 per cent of casein grew better than those on 10 per cent of casein, and they also had larger livers and kidneys. Accordingly the vitamin content per organ was greatest on the high protein diet, but the vitamin content per gm. of tissue was almost identical on the two levels of casein (Table VII).

Analyses after 5 weeks on the various diets yielded results very similar to those after 10 weeks, and the variations in the vitamin content of the kidneys and muscle tended to parallel those in the liver; these tissues contained roughly one-half and one-third, respectively, as much vitamin B₆ as the liver.

The failure of the high casein diet to alter the concentration of vitamin B₆ in rat tissue has a counterpart in the observation that the survival of the pyridoxine-deficient rat is affected only slightly, if at all, by an increase in dietary casein (9, 18). Young mice, on the contrary, lose their stored vitamin more rapidly (Table II) and have a higher mortality rate when the level of casein in the diet is increased.

TABLE VII

Effect of Casein upon Vitamin B₆ Content of Rat Tissues (Rats Depleted of Vitamin B₆ for 10 Weeks)

	Gain per wk.	Vitamin B ₆ per gm.			Vitamin B ₆ per organ	
		Liver	Kidneys	Muscle	Liver	Kidneys
	gm.	γ	γ	γ	γ	γ
Initial stores		10.6	6.5	5.6	40.7	5.5
10% casein, no B ₆	4	5.1	2.6	1.8	29	2.9
50% " " "	3	4.1	2.4	1.7	25.3	3.3
10% " 2.5 γ B ₆ per gm.	12	13.8	6.7	5.7	87	9.1
50% " 2.5 " " " "	20	14.0	5.3	5.0	149	11.9

SUMMARY

1. Young mice were fed diets deficient in vitamin B₆ and the tissue reserves of the vitamin found to diminish much more rapidly on 50 per cent of casein than when the diet contained 10 per cent of casein. Young mice were more sensitive to the protein than older ones. The mice on high protein lost more weight and had a higher mortality rate than those on the low protein diet.

2. The effect was not due to variation in caloric intake or to unequal urinary excretion of the vitamin or to the tryptophane content of the diets. Many of the severely deficient mice developed fatty livers.

3. The concentration of vitamin B₆ in the tissues increased progressively with increasing consumption of pyridoxine. At low levels of vitamin intake less vitamin was stored on the high protein diet. At relatively high levels of pyridoxine intake, however, the high protein diet favored the storage of the vitamin.

4. The level of casein in the diet did not appear to affect either the storage or the depletion of vitamin B₆ from the tissues of rats.

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PARTIAL SYNTHESIS OF COMPOUNDS RELATED TO ADRENAL CORTICAL HORMONES

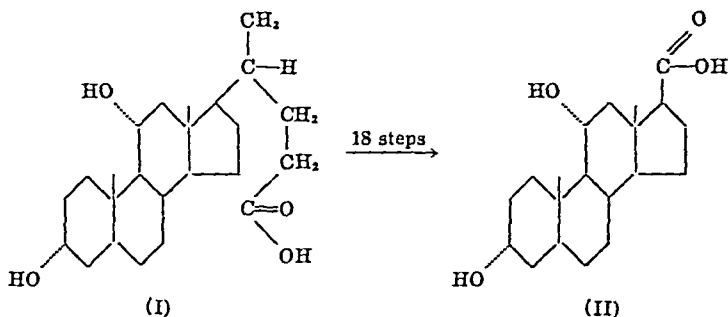
IX. STEPWISE DEGRADATION OF THE SIDE CHAIN OF 3(α),11(α)-DIHYDROXYCHOLANIC ACID*

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The present report deals with the stepwise removal of the side chain of 3(α),11(α)-dihydroxycholanolic acid I (1) and the preparation of the corresponding etio acid II.



The procedure devised by Barbier and Locquin (2) and applied first to the degradation of cholanolic acid by Wieland, Schlichting, and Jacobi (3) was employed. This method has been shown to be generally applicable to various hydroxylated bile acids, especially by Hoehn and Mason (4, 5) and by Reichstein and his colleagues (6-8). The pertinent literature is cited in the publications of these authors.

EXPERIMENTAL¹

3(α),11(α)-Diacetoxynorcholanyldiphenylethylene—20.0 gm. (0.05 mole) of methyl 3(α),11(α)-dihydroxycholanate (m.p. 126-130°) dissolved

* Part of the work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago. The expenses of part of the work were defrayed by a grant from Memorial Hospital, for which we wish to express our thanks.

¹ All melting points are corrected. The microanalyses were performed by Joseph Alicino (J. A.), The Squibb Institute for Medical Research, New Brunswick, New Jersey, by Dr. Adelbert Elek (Elek), The Rockefeller Institute for Medical Research,

in 650 ml. of anhydrous benzene were added to a solution of 20 mole equivalents (1.0 mole) of phenyl magnesium bromide (previously filtered to remove particles of metallic magnesium) in 400 ml. of ether and 500 ml. of benzene. The reaction mixture was heated under a reflux with stirring for 24 hours in an atmosphere of nitrogen, cooled, poured on ice, and acidified with 10 per cent sulfuric acid. The aqueous phase was separated and reextracted twice with ether. The organic solvent was washed with dilute hydrochloric acid, dilute sodium carbonate, and with water. The solvents were removed by distillation and the oily residue distilled with steam until 6.5 liters of distillate had been collected. The aqueous layer was decanted, the residual oil dissolved in 250 ml. of ethanol, 50 ml. of 4 N sodium hydroxide added, and the solution heated under a reflux for 15 minutes. 250 ml. of water were added and the alcohol removed by distillation. The residue was extracted with ether. The ether solution was extracted with 4 per cent sodium hydroxide, washed with water, dried over sodium sulfate, and the ether removed. The crude carbinol, after drying, was heated under a reflux for 1 hour with 150 ml. of glacial acetic acid; 25 ml. of acetic anhydride were added and heating was continued for another $\frac{1}{2}$ hour. The solution was cooled to 15° and 1.0 ml. of 70 per cent perchloric acid in 25 ml. of glacial acetic acid added.² After standing in an ice bath for 15 minutes, 5 ml. of water were added slowly to the chilled solution. The reaction mixture was then diluted with water and ether, neutralized with 10 per cent sodium carbonate, and extracted with ether. The ether was washed with water, dried over sodium sulfate, and the solvent removed. The residue crystallized from ethanol and 19.8 gm. of crude product melting at 127–133° were obtained. The second crop weighed 2.6 gm. and melted at 103–119°. The pure product, after recrystallization from ethanol, formed platelets melting at 148–149°; $[\alpha]_D^{24} = +21^\circ$ (CHCl₃).

C₄₀H₄₂O₄. Calculated, C 80.48, H 8.78; found (J. A.), C 80.42, H 8.88

From the alkaline hydrolysate of the crude carbinol, 3.2 gm. (15 per cent) of unchanged 3(α), 11(α)-dihydroxycholanic acid were isolated and purified as the methyl ester.

3(α), 11(α)-Dihydroxybisnorcholanyldiphenylethylene was obtained by alkaline hydrolysis of the diacetate. It crystallized in clusters of prisms from methanol, m.p. 177–179°; $[\alpha]_D^{23} = +35^\circ$ (CHCl₃).

C₃₆H₄₆O₂. Calculated, C 84.32, H 9.44; found (Elek), C 84.41, H 9.50

by John De Lucia (J. De L.), New York, and by Professor A. J. Haagen-Smit (H.-S.), California Institute of Technology. We wish to express our appreciation for this service.

² Schwenk, E., and Whitman, B., personal communication.

3(α),11(α)-Dihydroxynorcholanolic Acid—8.65 gm. of 3(α),11(α)-diacetoxybisanorcholanyldiphenylethylene were dissolved in 20 ml. of redistilled chloroform and 120 ml. of glacial acetic acid redistilled from CrO₃. 53.5 ml. of 3.26 N CrO₃ in 75 per cent acetic acid were acidified with a solution of 6 ml. of 10 per cent sulfuric acid in 20 ml. of redistilled glacial acetic acid and added dropwise with stirring at 1–3° over a period of 30 minutes. The excess CrO₃ was reduced with aqueous sodium bisulfite, and the reaction mixture buffered with sodium acetate and distilled with steam until 3 liters of distillate were collected. The non-volatile residue was extracted with ether-ethyl acetate 1:1 and washed with dilute hydrochloric acid and with water. The solvent was removed, the residue was dissolved in 250 ml. of ethanol, 50 ml. of 4 N sodium hydroxide were added, and the solution was heated under a reflux for 15 minutes. 500 ml. of water were added and the alcohol was removed. The aqueous alkaline residue was diluted with water, extracted with ether, and the combined ethereal extracts were counter-extracted twice with 4 per cent sodium hydroxide. The alkaline extracts were combined, covered with ether-ethyl acetate 1:1, and acidified with hydrochloric acid. The aqueous acid phase was extracted with ether-ethyl acetate mixture, washed with water, and dried over sodium sulfate. The product crystallized during removal of the solvent, the crude product weighing 3.2 gm. and melting at 217–223°. The mother liquors yielded four more crops totaling 770 mg. The pure acid, after recrystallization from ethyl acetate as long prisms, melted at 226–227°; $[\alpha]_D^{24} = +17^\circ$ (CHCl₃).

C₂₂H₃₄O₄. Calculated, C 72.98, H 10.10; found (J. De L.), C 72.98, H 10.20

For preparative purposes it is preferable to oxidize the crude oily 3(α),-11(α)-diacetoxybisanorcholanyldiphenylethylene directly without isolation of the intermediates. It was found advantageous to carry out the oxidation for from 3 to 5 hours at 0–3° and to isolate the product as before. Considerably better over-all yields were obtained in this way.

Methyl 3(α),11(α)-dihydroxynorcholanate, prepared with diazomethane, crystallized from acetone-petroleum ether as large transparent prisms melting at 158.5–160°; $[\alpha]_D^{25} = +15^\circ$ (CHCl₃).

C₂₂H₄₀O₄. Calculated, C 73.47, H 10.23; found (J. A.), C 73.51, H 10.33

3(α),11(α)-Diacetoxynorcholanolic acid can be isolated directly from the CrO₃ oxidation of 3(α),11(α)-diacetoxybisanorcholanyldiphenylethylene but the extraction from an organic solvent by aqueous base results in the formation of troublesome emulsions, since the sodium salt is very sparingly soluble in water. The compound is readily prepared by acetylation of the dihydroxy acid with acetic anhydride and HClO₄. The

pure acid crystallized from ethyl acetate-petroleum ether as slender prisms melting at 191–193°; $[\alpha]_D^{27} = +35^\circ$ (CHCl_3).

$\text{C}_{27}\text{H}_{42}\text{O}_6$. Calculated, C 70.11, H 9.15; found (J. A.), C 69.79, H 8.92

Methyl 3(α),11(α)-diacetoxynorcholanate, prepared with diazomethane, crystallized in platelets and melted at 131–132°; $[\alpha]_D^{27} = +44^\circ$ (CHCl_3).

$\text{C}_{28}\text{H}_{44}\text{O}_6$. Calculated, C 70.55, H 9.31; found (J. A.), C 70.65, H 9.21

3(α),11(α)-Dihydroxynorcholanyl Phenyl 24-Ketone—The neutral non-crystalline residues from the oxidation of several lots of crude 3,11-diacetoxynorcholanoyldiphenylethylene obtained from the Grignard reaction directly without isolation of intermediates were combined, saponified, and separated into ketonic and non-ketonic fractions by means of the Girard Reagent T. The ketonic fraction on standing in ethyl acetate-petroleum ether mixture deposited a crystalline product melting at 177–191°. Recrystallization from ethanol yielded clusters of tiny needles, m.p. 196–197.5°; $[\alpha]_D^{23} = +16^\circ$ (CHCl_3).

$\text{C}_{30}\text{H}_{44}\text{O}_6$. Calculated, C 79.60, H 9.80; found (Elek), C 79.48, H 9.79

3(α),11(α)-Diacetoxynorcholanyl phenyl 24-ketone was prepared by acetylation of the dihydroxy ketone with acetic anhydride and HClO_4 . Recrystallization from methanol and from ethanol yielded plates, m.p. 133–134°; $[\alpha]_D^{25} = +9^\circ$ (CHCl_3).

$\text{C}_{31}\text{H}_{46}\text{O}_6$. Calculated, C 76.08, H 9.01; found (Elek), C 76.12, H 9.10

3(α),11(α)-Diacetoxynorcholanoyldiphenylethylene—22.1 gm. (0.056 mole) of methyl 3(α),11(α)-dihydroxynorcholanate (m.p. 157–158°) were dissolved in 1000 ml. of hot anhydrous benzene and added to a solution of 27 mole equivalents (1.48 moles) of phenyl magnesium bromide, free of magnesium particles, in 600 ml. of ether and 500 ml. of benzene. The reaction was carried out and the product isolated in the same manner as with the bisnorcholanoyldiphenylethylene. The product crystallized directly from the acetic acid-acetic anhydride solution after addition of HClO_4 . The precipitation was completed by slow addition of water and the crude product recrystallized from ethanol. 26.4 gm. were obtained. The pure compound crystallized in platelets from ethanol and melted at 205–206°; $[\alpha]_D^{23} = +96^\circ$ (CHCl_3).

$\text{C}_{37}\text{H}_{50}\text{O}_4$. Calculated, C 80.37, H 8.65; found (Elek), C 80.57, H 8.65

From the alkaline hydrolysate of the crude carbinol 700 mg. of unchanged 3(α),11(α)-dihydroxynorcholanolic acid were isolated and purified as the methyl ester.

3(α),11(α)-Dihydroxynorcholanoyldiphenylethylene was obtained by

alkaline hydrolysis of the diacetate. It crystallized from ethyl acetate-petroleum ether in clusters of needles melting at $176.5\text{--}177^\circ$; $[\alpha]_D^{25} = +138^\circ$ (CHCl_3).

$\text{C}_{11}\text{H}_{14}\text{O}_2$. Calculated, C 84.29, H 9.30; found (Elek), C 84.33, H 9.45

3(\alpha),11(\alpha)-Dihydroxybisanorholanic Acid—27.97 gm. of *3(\alpha),11(\alpha)*-diacetoxyternorholanyldiphenylethylene were dissolved in 1750 ml. of glacial acetic acid redistilled from CrO_3 . 167 ml. of 3.46 N CrO_3 in 75 per cent acetic acid were acidified with a solution of 5.6 ml. of concentrated H_2SO_4 in 50 ml. of redistilled glacial acetic acid and added dropwise with stirring at $25\text{--}30^\circ$ over a period of 3 hours. Stirring was continued for an additional 4 hours. After chilling to 5° , the excess CrO_3 was reduced with aqueous sodium bisulfite. The reaction mixture was buffered with sodium acetate and distilled with steam until 9 liters of distillate were collected. The non-volatile fraction was extracted with ether and the ethereal solution, after being washed three times with dilute hydrochloric acid and with water, was evaporated to dryness. The oily residue was saponified with 230 ml. of 4 N NaOH in 650 ml. of ethanol under a reflux for 1 hour. 500 ml. of water were added and the alcohol distilled. The acid was isolated in the same manner as the nor acid, except that ether alone was used to extract the crude acid. Upon removal of the solvent crystallization occurred. The crude product melted at $252\text{--}256^\circ$ and 11.7 gm. were obtained. An additional 0.53 gm. of product was obtained from the mother liquors by conversion to the methyl ester and chromatographing on Al_2O_3 . The pure product after recrystallization from ethyl acetate-petroleum ether and from ether formed small transparent prisms which melted at $231\text{--}233^\circ$ and after cooling crystallized again and remelted at $254\text{--}257^\circ$. Rapid crystallization from hot concentrated solution in either ethyl acetate or acetone yielded irregular plates which melted at $258\text{--}260^\circ$. Both crystal forms had the same specific rotation, $[\alpha]_D^{25} = -8^\circ$ (ethanol).

$\text{C}_{22}\text{H}_{24}\text{O}_4$. Calculated, C 72.49, H 9.96; found (J. A.), C 72.48, H 9.93

Methyl 3(\alpha),11(\alpha)-dihydroxybisanorholanate, prepared with diazomethane, crystallized as platelets, m.p. $209\text{--}210^\circ$; $[\alpha]_D^{25} = -9^\circ$ (CHCl_3).

$\text{C}_{22}\text{H}_{24}\text{O}_4$. Calculated, C 72.98, H 10.10; found (J. De L.), C 73.20, H 10.31

3(\alpha),11(\alpha)-Diacetoxybisanorholanic Acid—This product is best prepared by acetylation of the dihydroxy acid in glacial acetic acid with acetic anhydride and perchloric acid, since like the nor acid it forms an insoluble sodium salt. The acetylation mixture was diluted with ether and water and washed with water to remove most of the acetic acid. The ethereal solution was extracted four times with 1.5 per cent sodium carbonate and twice with water. Each alkaline and water extract was introduced at once

into a hydrochloric acid-ice slurry covered with ether. The sodium salt is very sparingly soluble in water and a minimum of 500 ml. of 1.5 per cent sodium carbonate per gm. of the diacetoxy acid must be used to extract this acid from organic solvents if emulsions and precipitation are to be avoided. The aqueous acid phase was extracted with ether and reextracted twice with the same solvent. The ether extracts were washed with water, dried over sodium sulfate, and the solvent removed. The pure acid crystallized from ethyl acetate-petroleum ether, from ethanol, and from acetone-petroleum ether as glittering platelets melting at $211-212.5^{\circ}$; $[\alpha]_D^{25} = -22.5^{\circ}$ (CHCl_3).

$\text{C}_{26}\text{H}_{40}\text{O}_6$. Calculated, C 69.60, H 8.99; found (J. A.), C 69.57, H 9.02

Methyl 3(α),11(α)-diacetoxybisanorcholanate was prepared from the diacetoxy acid with diazomethane and crystallized from methanol as platelets melting at $136-138^{\circ}$; $[\alpha]_D^{23} = -16.2^{\circ}$ (CHCl_3).

$\text{C}_{27}\text{H}_{42}\text{O}_6$. Calculated, C 70.11, H 9.15; found (J. De L.), C 69.94, H 9.05

1,1-Diphenylmethyl-3(α),11(α)-dihydroxyetiocholan-yl)ethylene—22.5 gm. (0.06 mole) of methyl 3(α),11(α)-dihydroxybisanorcholanate were dissolved in 1500 ml. of hot anhydrous benzene and added to a solution of 20 mole equivalents (1.2 moles) of phenyl magnesium bromide, free of metallic magnesium, in 450 ml. of ether and 500 ml. of benzene. The reaction mixture was heated under a reflux with stirring for 45 hours in an atmosphere of nitrogen, and the crude carbinol isolated as in the previous experiments. 1.6 gm. (7 per cent) of unchanged 3(α),11(α)-dihydroxybisanorcholanate were recovered. Dehydration of the carbinol was effected by the continuous distillation of a benzene solution with 600 mg. of iodine for 4 hours. 52 per cent of the calculated amount of water was measured in the distillate. The benzene solution was diluted with ethyl acetate, washed with sodium thiosulfate and with water, dried over sodium sulfate, and the solvents removed by distillation. The residue crystallized from ethyl acetate and 14.54 gm. of crude product, melting at $229-231^{\circ}$, were obtained in the first crop. The mother liquors crystallized poorly but on chromatographing on Al_2O_3 , an additional 3.87 gm. of crystalline product were obtained. Recrystallization from ethanol yielded slender prisms melting at $234-235.5^{\circ}$; $[\alpha]_D^{24} = +285^{\circ}$ (CHCl_3).

$\text{C}_{31}\text{H}_{44}\text{O}_2$. Calculated, C 84.25, H 9.17; found (J. A.), C 84.24, H 9.03

1,1-Diphenylmethyl-3(α),11(α)-diacetoxyetiocholan-yl)ethylene was prepared by acetylation of the dihydroxy compound with acetic anhydride and HClO_4 and crystallized from methanol, m.p. $186.5-187.5^{\circ}$; $[\alpha]_D^{26} = +206^{\circ}$ (CHCl_3).

$\text{C}_{33}\text{H}_{48}\text{O}_4$. Calculated, C 80.24, H 8.51; found (J. A.), C 79.93, H 8.39

3(α),11(α)-Diacetoxy-20-ketopregnane—5.0 gm. of 1,1-diphenylmethyl-3(α),11(α)-diacetoxyetiocholanyl)ethylene were dissolved in 400 ml. of anhydrous methanol-ethyl acetate 1:1.³ The solution was chilled to -30° and 5 mole equivalents of ozone in a 6 per cent stream were passed through the solution. The solution was transferred to a chilled hydrogenation vessel and shaken in an atmosphere of hydrogen with 5.0 gm. of a 5 per cent palladium-calcium carbonate catalyst until the uptake of hydrogen ceased. This was generally effected in 15 minutes. The catalyst was filtered, and the solvent was removed by distillation. The light yellow mass crystallized readily but it was found preferable to separate the product into ketonic and non-ketonic fractions by means of the Girard Reagent T. 3.8 gm. of ketonic material were obtained which was crystallized from ethyl acetate as prisms, m.p. 147–148.5°; $[\alpha]_D^{25} = +74^{\circ}$ (CHCl_3). 2.79 gm. were obtained (76 per cent).

$\text{C}_{27}\text{H}_{44}\text{O}_4$. Calculated, C 71.74, H 9.15; found (H.-S.), C 71.82, H 9.14

3(α),11(α)-Dihydroxy-20-ketopregnane was prepared from the diacetate by hydrolysis at room temperature with 2 N NaOH in 75 per cent ethanol. The product was crystallized from ethyl acetate and ethyl acetate-petroleum ether as platelets, m.p. 182.5–184°; $[\alpha]_D^{23} = +96^{\circ}$ (CHCl_3).

$\text{C}_{27}\text{H}_{44}\text{O}_4$. Calculated, C 75.40, H 10.25; found (J. A.), C 75.53, H 9.97

3,11,20-Triketopregnane—175 mg. of 3(α),11(α)-dihydroxy-20-ketopregnane were dissolved in 5.0 ml. of glacial acetic acid which had been redistilled from CrO_3 , the solution was chilled until incipient crystallization, and 208 mg. of CrO_3 in 8.0 ml. of 75 per cent acetic acid were added. The mixture was stored at 4° for 16 hours and the excess CrO_3 was reduced with sodium bisulfite. The solution was poured into water and extracted thoroughly with ether. The ether solution was washed with dilute sulfuric acid, dilute sodium bicarbonate solution, and with water, dried over sodium sulfate, and the ether removed. The residue was crystallized from ether and from ethyl acetate-petroleum ether as long needles, m.p. 157.5–159°; $[\alpha]_D^{26} = +121^{\circ}$ (acetone). Hegner and Reichstein (9) report a melting point of 154–156° and $[\alpha]_D^{20} = +119.5^{\circ}$ (acetone).

$\text{C}_{27}\text{H}_{44}\text{O}_4$. Calculated, C 76.32, H 9.15; found (J. A.), C 76.20, H 9.16

3(α),11(α)-Dihydroxy-20-keto-21-benzalpregnane—1.0 gm. of 3(α),11(α)-dihydroxy-20-ketopregnane was dissolved in 15 ml. of absolute ethanol and chilled to -15° , care being taken to exclude moisture. 15 ml. of a 3 per cent sodium ethoxide solution chilled to 0° were added and the mixture

³ This solvent mixture was suggested to us by Dr. Kendall and Dr. Turner of the Mayo Foundation. It has proved highly satisfactory in various ozonolyses and we wish to express our appreciation for the suggestion.

chilled to -15° . 1.0 ml. of cold (0°) freshly redistilled benzaldehyde was slowly added and the reaction mixture, immersed in an ice-salt bath, was allowed to warm to -3° in the course of 18 hours. The solution was poured into ice water and ether, and reextracted twice with ether. The ether solution was washed with water and, after drying with sodium sulfate, the ether was removed and the crude product crystallized as white needles retaining some benzaldehyde. The product was recrystallized from ethyl acetate, yielding 1.05 gm. with a melting point of $215-217^{\circ}$. The pure product crystallized from benzene and from acetone as clumps of needles, m.p. $219-220^{\circ}$; $[\alpha]_D^{24} = +107^{\circ}$ (absolute ethanol); $+86^{\circ}$ (CHCl_3).

$\text{C}_{23}\text{H}_{33}\text{O}_3$. Calculated, C 79.57, H 9.06; found (H.-S.), C 79.98, H 8.96

3(\alpha),11(\alpha)-Diacetoxy-20-keto-21-benzalpregnane—250 mg. of *3(\alpha),11(\alpha)*-dihydroxy-20-keto-21-benzalpregnane were acetylated by heating with 2.0 ml. of acetic anhydride and 0.25 ml. of pyridine for 1 hour. The reaction mixture was poured into water and after standing for a short interval crystallized. It was recrystallized from ethyl acetate-petroleum ether as long needles, m.p. $162-163^{\circ}$; $[\alpha]_D^{24} = +55^{\circ}$ (absolute ethanol); $+71^{\circ}$ (CHCl_3).

$\text{C}_{22}\text{H}_{32}\text{O}_6$. Calculated, C 75.85, H 8.35; found (H.-S.), C 75.86, H 8.52

3(\alpha),11(\alpha)-Dihydroxyetiocholanolic Acid—0.97 gm. of *3(\alpha),11(\alpha)*-diacetoxy-20-keto-21-benzalpregnane (1.92 millimoles, m.p. $162-163^{\circ}$) was dissolved in 400 ml. of anhydrous methanol-ethyl acetate 1:1, the solution was chilled to -45° , and 8 mole equivalents of ozone were passed through the solution in a 6 per cent stream. The solution was transferred to a chilled hydrogenation vessel and shaken in an atmosphere of hydrogen with 5.0 gm. of a 5 per cent palladium-calcium carbonate catalyst until the uptake of hydrogen had ceased. The catalyst was filtered and the solvent removed by distillation under diminished pressure. The yellowish gummy residue was dissolved in 45 ml. of redistilled ethanol, 9.6 ml. of 2 N periodic acid were added, and the solution stored at room temperature for 40 hours. The reaction mixture was diluted with water and the alcohol removed by distillation. The residue was extracted with ether, and the ethereal solution washed with dilute brine and then three times with 1 N sodium hydroxide solution. The alkaline solution was heated on the steam bath for 1 hour and acidified with dilute hydrochloric acid and extracted thoroughly with ether. The ether extracts were combined, washed with dilute brine, and dried over sodium sulfate. After removal of the solvent the residue was crystallized from benzene and from dilute ethanol as long needles and from ethyl acetate as clusters of soft needles, m.p. $250-251^{\circ}$; $[\alpha]_D^{24} = +60^{\circ}$ (95 per cent ethanol). The compound holds solvent tenaciously and in-

variably gave low carbon values despite exhaustive drying. 485 mg. were obtained (75 per cent).

$C_{21}H_{32}O_4$. Calculated, C 71.39, H 9.59; found (Elek), C 70.76, H 9.53

Methyl 3(α),11(α)-dihydroxyetiocholanate was prepared from the acid with diazomethane. It crystallized from ethyl acetate-petroleum ether mixtures as clusters of tiny needles, m.p. 159–160°; $[\alpha]_D^{25} = +55.6^\circ$ ($CHCl_3$).

$C_{21}H_{32}O_4$. Calculated, C 71.96, H 9.78; found (J. A.), C 71.99, H 9.57

Methyl 3(α),11(α)-diacetoxyetiocholanate was prepared from the methyl ester by acetylation with acetic anhydride, perchloric acid being used as catalyst. Crystallized from ethyl acetate-petroleum ether it formed needles, m.p. 169.5–171°; $[\alpha]_D^{25} = +46.3^\circ$ ($CHCl_3$).

$C_{23}H_{34}O_6$. Calculated, C 69.10, H 8.81; found (H.-S.), C 69.24, H 9.09

DISCUSSION

In a series of reactions involving eighteen steps it is not expected that the over-all yield will be high, although the yield in the individual operations may be satisfactory. In the present investigation only one yield lower than 70 per cent of calculated was encountered. This was the conversion of the bisnor ester to the 1,1-diphenylmethyl-3(α),11(α)-dihydroxyetiocholanylethylene, when 64 per cent was obtained. Nevertheless the over-all yield, if saponifications and esterifications are disregarded, was only slightly in excess of 9 per cent and even this figure could not be achieved without resort to chromatographic purification. Although these figures are higher than the majority of results recorded in the literature, it is obvious that a more efficient procedure is highly desirable and that the present methods were justifiable only on the ground that they were the only ones available. The recent experiments of Meystre, Frey, Wettstein, and Miescher (10) and of Meystre, Ehmann, Neher, and Miescher (11) indicate that a great improvement in yield to the stage of the 20-ketopregnane derivative can be achieved with desoxycholic acid over the methyletiocholanylethylene derivative and further reports on the extension of this procedure will be of great interest.

Meystre *et al.* (10) have recorded the absorption spectrum of 3,12-diacetoxymethylnorcholesterdiphenylethylene. Fig. 1 offers a comparison of the ultraviolet absorption curves for the three diphenylethylenes described in this report. It is interesting to note the very marked decrease in intensity of the absorption for the diphenylmethyletiocholanylethylene (Curve 3) as contrasted with either the tennor- or bisnordiphenylethylenes (Curves 1 and 2). Together with this diminution there is a definite shift of the absorption maximum toward shorter wave-length. These changes in intensity and

wave-length of the maximum are very probably explained by steric effects, since the presence of both the methyl and etiocholanyl groups on the diphenylethylene chromophore would markedly increase the strain in the coplanar configuration and thereby inhibit the resonance of the compound. This effect has been recently discussed by Remington (12), Jones (13), Rodebush and coworkers (14, 15), and by other authors cited in these publications. Our results are in accord with their generalizations.

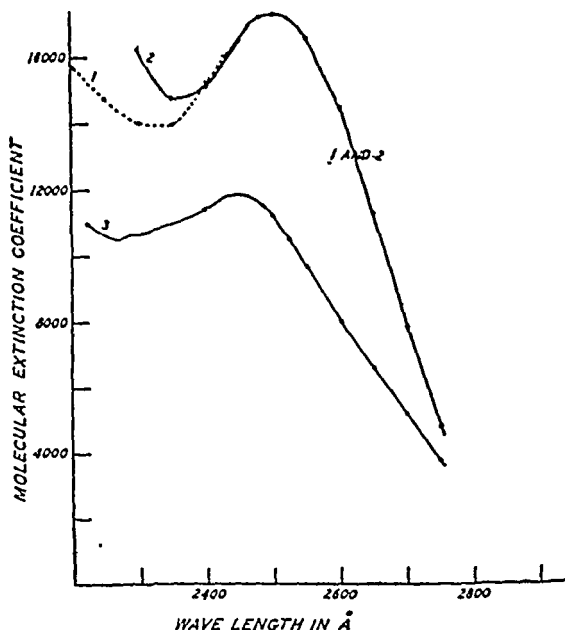


FIG. 1. Absorption spectra of steroid diphenylethylenes. Curve 1, 3(α), 11(α)-diacetoxybisnorcholanyldiphenylethylene, $\epsilon_{2550} = 17,200$; Curve 2, 3(α), 11(α)-diacetoxyternorcholanyldiphenylethylene, $\epsilon_{2550} = 17,200$; Curve 3, 1,1-diphenylmethyl-3(α), 11(α)-diacetoxyetiocholanylethylene, $\epsilon_{2550} = 11,800$. The solvent was 95 per cent ethanol.

The 3(α), 11(α)-dihydroxynorcholanyl phenyl 24-ketone isolated in these experiments from the Grignard addition to an ester is similar to the series of products prepared by Hoehn and Moffett (16) by interaction of diphenyl cadmium with the acid chlorides of bile acids. It does not constitute any considerable portion of the reaction product isolated after dehydration and oxidation. Since the mechanism of reaction between esters and the Grignard reagent is not certain (17), it cannot be decided whether the ketone is a direct intermediate in the formation of the carbinol or is formed by secondary processes. It is perhaps of significance that the Grignard addi-

tion to the 3(α),11(α)-dihydroxycholanolic acid is a relatively sluggish reaction characterized by the formation of considerable insoluble material. This in part accounts for the comparatively low yield in the first step of the degradation and the insolubility of the intermediate may explain the formation of the ketone in the amount isolated. The absorption spectrum of 3(α),11(α)-diacetoxynorcholanyl phenyl 24-ketone is shown in Fig. 2 together with the analogous compound derived from cholanolic acid.⁴

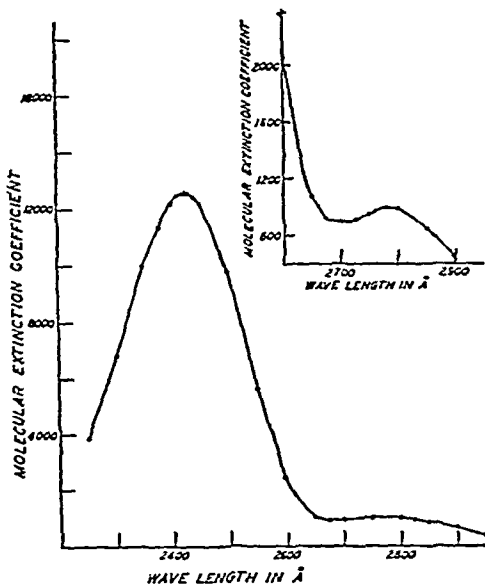
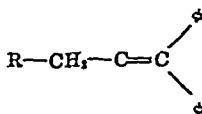


FIG. 2. Absorption spectrum of 3(α), 11(α)-diacetoxynorcholanyl phenyl 24-ketone, $\epsilon_{24125} = 12,500$; $\epsilon_{28120} = 980$. The spectrum of norcholanyl phenyl 24-ketone is identical, $\epsilon_{24125} = 12,800$; $\epsilon_{28120} = 1030$. The solvent was 95 per cent ethanol.

The oxidation of a diphenylethylene of the type



⁴ This sample was furnished us by Dr. Willard Hoehn. We wish to express our thanks for this cooperation.

could yield $R-COOH$ if, as should be possible, the carbon atom α to the ethylene were oxidized to a ketone group before fission of the ethylenic bond. After carefully examining the mother liquors from several lots of 3(α),11(α)-dihydroxynorcholanic acid, we were able to isolate a small quantity of the corresponding bisnor acid. A few attempts were made to increase the yield of the bisnor acid by modifying the oxidation of the diphenylethylene but we were unable to obtain any appreciable yield of the C_{22} acid. However, this reaction may account in part at least for the sometimes disappointing yields in the oxidation of the diphenylethylenes with chromic acid.

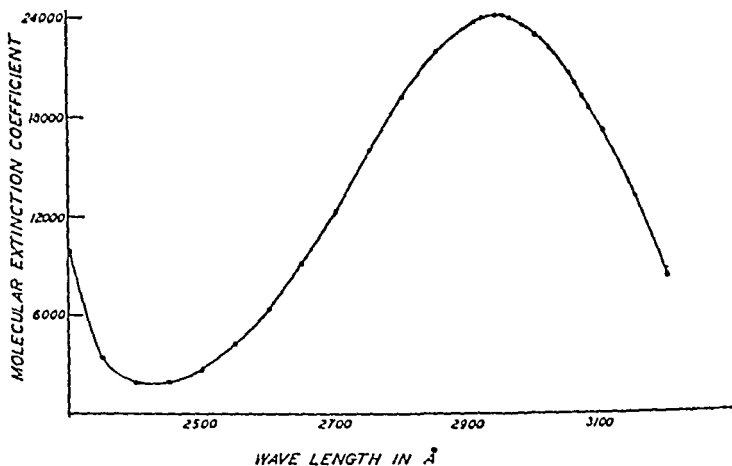


FIG. 3. Absorption spectrum of 3(α),11(α)-diacetoxy-20-keto-21-benzalpregnane, $\epsilon_{29.0} = 24,000$. The solvent was 95 per cent ethanol.

The absorption spectrum of 3(α),11(α)-diacetoxy-20-keto-21-benzalpregnane is given in Fig. 3. The spectrum of this substance and related derivatives, especially the 3,12-diacetoxy compound, furnishes a convenient method for determining the homogeneity of the product. The ketone group is quite reactive and readily forms enol acetates. When a difficultly acetyltable hydroxyl group is present, for example the case of the 12(α)-hydroxyl group of desoxycholic acid, the relatively vigorous conditions necessary to effect acetylation lead to the simultaneous formation of variable amounts of enol acetate. The latter is readily detected by the higher absorption maximum and distinct shift toward the longer wave-length. The acetylation can be more readily achieved by the excellent perchloric acid-catalyzed reaction of Schwenk and Whitman² but here also it is necessary to conduct the reaction between -10° and 0° for a relatively short time. More prolonged treatment, especially at room temperature, yields

up to 20 to 30 per cent enol acetate. With the more readily esterified 11(α)-hydroxyl group of the compound described in this report no difficulty is encountered with the formation of enol acetates as an undesirable side product.

SUMMARY

The degradation of the side chain of 3(α),11(α)-dihydroxycholelanic acid to 3(α),11(α)-dihydroxyetiocholelanic acid has been described. All the intermediate compounds have been isolated and characterized and the ultraviolet absorption spectra of five of these have been presented.

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PARTIAL SYNTHESIS OF COMPOUNDS RELATED TO ADRENAL CORTICAL HORMONES

X. PREPARATION OF 3(α),11(α)-DIHYDROXYETIOCHOLANIC ACID FROM ETIODESOXYCHOLIC ACID*

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For the partial synthesis of adrenal cortical hormones and related substances it was desirable to prepare derivatives of etiocholanolic acid with hydroxyl groups at positions 3 and 11 of the steroid nucleus. This can be effected by the stepwise degradation of 3(α),11(α)-dihydroxycholanolic acid (1) or by the introduction of the 11-hydroxyl group directly into the etio acid. Under the latter circumstance the considerable losses incurred in the removal of the side chain are sustained at the expense of the commercially available desoxycholic acid. Since, however, etio-desoxycholic acid was unavailable at the time the investigations reported in the previous paper were carried out, and since the difficulties involved in degrading 3,11-dihydroxycholanolic acid were not anticipated to be greater than those encountered with desoxycholic acid, the stepwise degradation of the 3,11-dihydroxy acid was accomplished before the alternative procedure was attempted. Through the assistance of Ciba Pharmaceutical Products, Inc., George A. Breon and Company, and Charles E. Frosst and Company, generous gifts of etio-desoxycholic acid have made the approach from this compound feasible. It is a pleasure to acknowledge their assistance.

It was found that bromination α to the 12-keto group in the etio acid as well as hydrolysis of the bromo keto ester was much more readily effected than with the corresponding derivatives of cholanolic acid. While bromination of methyl 3(α)-acetoxy-12-ketocholanate at room temperature is a slow reaction requiring days even in the presence of HBr, the bromination of the corresponding etio ester was complete in about 1 to 2 hours with small concentrations of HBr as catalyst. This effect can be explained on the basis of steric hindrance exerted by the side chain of the cholanolic acid, since to all practical purposes halogen can add to the enol of the 12-keto ester from but one direction (2). An alternative explanation is that in the etio acid the ketone group at C-12 enolizes more readily by virtue of the ac-

* Part of the work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago. The expenses of part of the work were defrayed by a grant from Memorial Hospital, for which we wish to express our thanks.

tivating influence of the carboxyl group in the γ position at C-17. Probably both mechanisms operate in the bromination reaction. Unfortunately the bromo keto esters were not obtained in crystalline state and it is therefore impossible to compare the yield of the two bromo compounds isomeric at C-11 with the respective products from the bromination of the 12-keto-cholanic acid derivatives, where the 11(α)-bromo ester is practically the sole product of the reaction.

The etio acid and the cholanic acid also differ in the rate of alkaline hydrolysis of the 11-bromo-12-keto esters. The more readily hydrolyzed methyl 3(α)-acetoxy-11(β)-bromo-12-ketocholanoate requires about 3 hours with 0.5 *N* base at room temperature for hydrolysis of the halogen (2), while the hydrolysis of the 11(α)-bromo epimer requires a considerably longer time. On the other hand the hydrolysis of halogen from the 11-bromo-12-ketoetiocholanoate is complete with 0.2 *N* base in less than 20 minutes at room temperature. This striking difference is more readily explained by the influence of the adjacent carboxyl group at C-17 on the substituents in Ring C than by purely steric considerations. Additional evidence for the markedly increased activation of the Ring C carbonyl group is afforded by the ease with which rearrangement of the 11-hydroxy-12-keto ketol structure takes place in the etio acid compared with the cholanic acid. With 2 *N* NaOH at room temperature for 62 hours the etiocholanic acid derivative rearranges completely from the 11-hydroxy-12-keto structure to the isomeric 11-keto-12-hydroxy compound. With the corresponding cholanic acid compound the rearrangement of the ketol structure does not take place to any considerable extent except at elevated temperatures. It appears therefore that the tendency of the ketol structure in Ring C to assume the more stable 11-keto-12-hydroxy configuration is a dominant feature of the etio acid derivatives. The failure of 3(α),12(α)-dihydroxy-11-ketoetiocholanic acid to form an oxime and to undergo Wolff-Kishner reduction, at least in part, to 3(α),11(α)-dihydroxyetiocholanic acid is similar to that of the corresponding cholanic acid derivative (3, 4). On the other hand the 3,11-dihydroxy-12-ketocholanic and etiocholanic acids form ketonic derivatives and yield 3(α),11(α)-dihydroxy acids upon Wolff-Kishner reduction, as has been shown for the cholanic acid derivatives (2, 5) and for the etio acid in this report.

Riegel and Moffett (6) have described a compound which, in agreement with the older literature, they designated 3,11-dihydroxy-12-ketoetiocholanic acid. Their product unquestionably was 3,12-dihydroxy-11-ketoetiocholanic acid, since it had been subjected to hydrolysis with base at elevated temperature. Only one of their fractions, m.p. 242-250°, had a specific rotation (+87° in ethanol) as high as that recorded here (+88° in acetic acid) for what is believed to be the same compound which melted at

275–277°. The discrepancy can hardly be due to incompleteness of isomerization and may perhaps be due to solvation of the crystals. It is apparent from their results as well as ours that the melting point of this compound is not a satisfactory criterion of purity. The product can, however, be satisfactorily purified over the methyl ester diacetate.

EXPERIMENTAL¹

Methyl 3(α)-Acetoxy-12-ketoetiocholanate—3(α)-Hydroxy-12-ketoetiocholanolic acid was prepared by the method of Schwenk, Riegel, Moffett, and Stahl (7), esterified with diazomethane, and acetylated with acetic anhydride and HClO_4 .² Crystallization from ethyl acetate yielded flat plates, m.p. 157–158°; $[\alpha]_D^{25} = +152^\circ$ (CHCl_3).

$\text{C}_{22}\text{H}_{34}\text{O}_6$. Calculated, C 70.74, H 8.78; found (H.-S.), C 70.59, H 8.99

3(α),12-Dihydroxy-11-ketoetiocholanolic Acid—3.26 gm. of methyl 3(α)-acetoxy-12-ketoetiocholanate were dissolved in 20 ml. of glacial acetic acid and 8.25 ml. of 2.24 N Br_2 in glacial acetic acid added. A small amount of dry HBr in acetic acid was added and the solution stored in the dark for 18 hours. The substitution was complete at the end of 2 hours. The solution was poured into ether, washed thoroughly with water, and the ether removed under diminished pressure at a bath temperature not exceeding 40°. The residue was dissolved in 35 ml. of ethanol and 35 ml. of 4.2 N NaOH added. After standing at room temperature for 62 hours, the solution was added dropwise with vigorous stirring to an excess of dilute H_2SO_4 at 33°. The precipitate was completely crystalline. Recrystallization from ethanol yielded 1.4 gm. of prisms which softened slightly at 256° and melted at 281°. The mother liquors upon concentration yielded 800 mg. of stout prisms which melted at 277° with some preliminary softening. Both products had identical optical rotations. The melting point of this compound varies considerably with the rate of heating and with the size of the crystalline aggregates. The purest product melted at 275–277° with rapid heating and foamed at 279–281°; $[\alpha]_D^{25} = +88^\circ$ (acetic acid).

$\text{C}_{22}\text{H}_{34}\text{O}_6$. Calculated, C 68.54, H 8.63; found (H.-S.), C 68.64, H 8.76

110 mg. of the product were heated under a reflux for 2 hours in 5.0 ml. of 4 N aqueous NaOH . The acid recovered from this treatment was identi-

¹ All melting points are corrected. The microanalyses were performed by Professor A. J. Haagen-Smit (H.-S.), California Institute of Technology, by Dr. T. S. Ma (T. S. M.), Department of Chemistry, University of Chicago, and by Joseph Alicino (J. A.), The Squibb Institute for Medical Research, New Brunswick, New Jersey. I wish to express my appreciation for this service.

² Schwenk, E., and Whitman, B., personal communication.

cal in melting point and specific rotation with the starting product and yielded the same methyl ester diacetate (melting point of pure compounds and of mixtures).

The free acid failed to form an oxime when heated with hydroxylamine acetate in ethanol for 3 hours.

Methyl 3(α),12-dihydroxy-11-ketoetiocholanate was prepared with diazomethane. The product was recrystallized from dilute methanol and from acetone-petroleum ether as prisms, m.p. 137.5–139°; $[\alpha]_D^{25} = +88^\circ$ (CHCl_3).

$\text{C}_{21}\text{H}_{32}\text{O}_6$. Calculated, C 69.20, H 8.85; found (J. A.), C 69.11, H 8.96

Methyl 3(α),12-diacetoxy-11-ketoetiocholanate was prepared from the methyl ester by esterification with acetic anhydride and HClO_4 . It was recrystallized from petroleum ether as needles, m.p. 134.5–135.5°; $[\alpha]_D^{24} = +81^\circ$ (CHCl_3).

$\text{C}_{23}\text{H}_{34}\text{O}_7$. Calculated, C 66.94, H 8.09; found (H.-S.), C 67.20, H 8.26

"*Wolff-Kishner Reduction*" of *3(α),12-Dihydroxy-11-ketoetiocholanate*—1.1 gm. of the acid with 3.0 ml. of 100 per cent hydrazine hydrate in 10 ml. of absolute ethanol were heated under a reflux for 3 hours and the solution transferred to a bomb tube with 60 ml. of sodium ethylate prepared from 3 gm. of sodium. The bomb was sealed and heated for 5 hours from 190–210°. The contents were removed, acidified, and extracted with ether. The ether solution yielded as the principal product (588 mg.) a compound melting at 250–253°. The remainder of the product was an oil which was not further investigated. The crystalline fraction after recrystallization from acetone-petroleum ether mixture and from acetone melted at 254–260°; $[\alpha]_D^{25} = +54^\circ$ (acetone).

$\text{C}_{20}\text{H}_{28}\text{O}_5$. Calculated, C 68.15, H 9.15; found (T. S. M.), C 68.75, H 9.42

The methyl ester, prepared with diazomethane, melted at 172–175°.

The ester was acetylated with acetic anhydride and HClO_4 . The methyl triacetate ester obtained upon crystallization from petroleum ether melted at 196–199° after softening at about 159°; $[\alpha]_D^{21} = +76^\circ$ (CHCl_3). It gave no depression of the melting point when admixed with the methyl 3(α),11,12-triacetoxyetiocholanate described later in this report. It seems probable that both this product and that subsequently described are mixtures of epimeric triacetates in which the 11-hydroxyl group is α , since the product can be completely acetylated. This conclusion should be regarded as tentative, since the influences of the hydroxyl group at C-12 and the carboxyl group at C-17 have not been investigated thoroughly enough to base configurational designation upon the ease of acetylation in the etio acids.

Methyl 3(α),11(α ?)-Diacetoxy-12-ketoetiocholanate—Preliminary experi-

ments had shown that bromination of methyl 3(α)-acetoxy-12-ketoetiocholanate yielded amorphous products even after reesterification and chromatographic separation. One experiment yielded a crystalline product, m.p. 135–136°, but the amount obtained was too small for further characterization. The bromination product, obtained from 2.8 gm. of methyl 3(α)-acetoxy-12-ketoetiocholanate by the method previously described, was esterified with diazomethane and acetylated with acetic anhydride and HClO_4 . The product was dissolved in 200 ml. of redistilled ethanol, 45 ml. of 1.04 N NaOH were added, and the volume of the solution made to 250 ml. with ethanol. Samples were removed at intervals, acidified with HNO_3 , an excess of standard AgNO_3 added, and the solution titrated with NH_4SCN , with $\text{Fe}_2(\text{SO}_4)_3$ as indicator. The halogen was completely ionic after 20 minutes at room temperature (23°). The remainder of the solution was acidified with dilute HNO_3 , extracted with ether, washed with water, and the solvent removed at room temperature. The residue was esterified with diazomethane and acetylated with acetic anhydride and HClO_4 . The product was crudely separated on 35 gm. of Al_2O_3 into two principal fractions by elution with 500 ml. of petroleum ether-benzene (4:1) and 500 ml. of ether. These solvents respectively removed 1.9 gm. of an amorphous gum and 811 mg. of crystalline product. An additional 200 mg. eluted with ethyl acetate were discarded. The crystalline eluate was recrystallized from petroleum ether with a small amount of acetone and long needles melting at 173.5–174.5° were obtained; $[\alpha]_D^{24} = +173^\circ (\text{CHCl}_3)$.

$\text{C}_{23}\text{H}_{34}\text{O}_7$. Calculated, C 66.94, H 8.09; found (H.-S.), C 67.07, H 8.31

Methyl 3(α),11(α)-Diacetoxyetiocholanate—610 mg. of methyl 3(α),11(α ?)-diacetoxy-12-ketoetiocholanate were dissolved in 5.0 ml. of absolute ethanol, 2.0 ml. of 100 per cent hydrazine hydrate added, and the solution stored overnight at room temperature. After transferring to a bomb tube with 100 ml. of sodium ethylate prepared from 10 gm. of sodium, the bomb was sealed and heated at 190–200° for 2 hours. The contents were acidified, extracted with ether, and the acid fraction esterified with diazomethane and acetylated with acetic anhydride and HClO_4 . The product was freed of a small amount of colored impurity with Al_2O_3 and the residue weighed 410 mg. Crystallization yielded 45 mg. melting at 165–167°, which gave no depression upon admixture with an authentic specimen of methyl 3(α),11(α)-diacetoxyetiocholanate (m.p. 169°). From the mother liquors upon chromatographing an additional 40 mg. of authentic methyl 3(α),11(α)-diacetoxyetiocholanate were obtained. No other crystalline product was isolated.

The amorphous product of hydrolysis of the bromo keto ester (1.9 gm.) was subjected to Wolff-Kishner reduction in the same manner previously

described and the product was esterified and acetylated as before. By chromatographing on Al_2O_3 , 150 mg. of pure methyl 3(α),11(α)-diacetoxyetiocholanate were obtained together with considerable material which appeared to be mixed crystals of this substance and the methyl ester triacetate described in the following section.

Methyl 3(α),11,12-Triacetoxyetiocholanate—Several crystalline fractions which showed similar melting behavior (beginning of melting observed at 165–173° but a clear melt obtained at about 193°) were combined and recrystallized from petroleum ether. Several crystallizations yielded a product which softened at 175° and gave a clear melt at 193–197°; $[\alpha]_D^{22} = +75^\circ$ (CHCl_3).

$\text{C}_{27}\text{H}_{46}\text{O}_8$. Calculated, C 65.83, H 8.19; found (H.-S.), C 65.55, H 8.41

It is probable that this fraction was a mixture of stereoisomeric trihydroxy esters.

SUMMARY

1. 3(α),11(α)-Dihydroxyetiocholanic acid has been prepared from etio-desoxycholic acid by a procedure similar to that previously found successful with desoxycholic acid.

2. Methyl 3(α)-acetoxy-12-ketoetiocholanate brominates more readily at C-11 than the corresponding derivative of cholanic acid.

3. The halogen of methyl 3(α)-acetoxy-11-bromo-12-ketoetiocholanate is rapidly hydrolyzed with dilute base at room temperature and the product upon Wolff-Kishner reduction is converted to a mixture from which methyl 3(α),11(α)-diacetoxyetiocholanate and methyl 3(α),11,12-triacetoxyetiocholanate have been isolated.

4. 3(α),11-Dihydroxy-12-ketoetiocholanic acid is converted to 3(α),12-dihydroxy-11-ketoetiocholanic acid upon standing in 2 N base at room temperature.

5. 3(α),12-Dihydroxy-11-ketoetiocholanic acid, like the corresponding cholanic acid derivative, does not form an oxime and upon "Wolff-Kishner reduction" yields a mixture in which 3,11,12-trihydroxyetiocholanic acids predominate.

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THE RELATION OF THE THYROXINE CONTENT OF THE THYROID GLAND AND OF THE LEVEL OF PROTEIN-BOUND IODINE OF PLASMA TO IODINE INTAKE*

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The factors that influence the storage of thyroxine in the thyroid gland have been under investigation in this laboratory (1). In the present study, measurements were made of the amounts of thyroxine and total iodine contained in the gland and of total and protein-bound iodine in plasma of rats whose daily iodine intakes were varied from 1 to 2 γ to as much as 480 γ . The levels of protein-bound iodine of plasma were found to be related to the thyroxine content of the gland and both, in turn, were dependent upon iodine intake.

EXPERIMENTAL

70 male rats weighing from 190 and 268 gm. were selected for this experiment. They had been maintained on a diet (No. 1) of 68.5 per cent wheat, 5 per cent casein, 10 per cent fish meal, 10 per cent alfalfa, 1.5 per cent sodium chloride, and 5 per cent sardilene, a fish oil that contained 400 A. O. A. C. chick units of vitamin D and 3000 U. S. P. units of vitamin A per gm.

The rats were divided into seven groups, A to G. All except Group B were placed on a diet (No. 2) that had the following composition: 67.5 per cent whole wheat, 15 per cent casein, 10 per cent whole milk powder, 1 per cent NaCl, 1.5 per cent chalk, 5 per cent hydrogenated vegetable oil, and 50 cc. of sardilene per 8 kilos of diet.

Group A received Diet 2, which contained approximately 0.1 γ of iodine per gm. of diet. Groups C, D, E, F, and G were fed Diet 2 fortified with KI to contain respectively 1, 5, 10, 20, and 30 γ of iodine per gm. of diet. Group B continued to receive Diet 1, which contained 0.3 γ of iodine per gm. of diet. The diets fed to Groups C, D, E, F, and G were mixed with the required quantity of KI in a ball mill to insure thorough mixing.

Each diet was fed for 22 to 24 days. Iodine-free distilled water was provided for drinking purposes. In order to permit the measurement of daily food intake, each rat (except those of Group B) was kept separately in a metabolism cage.

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The rats were fasted for 24 hours before removal of blood and excision of the thyroid gland for iodine analyses. The plasma of two rats was pooled in order to provide enough material for both total and protein-bound iodine determinations on the same plasma sample. In some cases, the thyroids of the same two rats were also pooled before analysis.

The methods used for determining plasma iodine and the thyroxine content of the gland have been reported elsewhere (2).

Results

Thyroid Iodine—The effects of increasing iodine intake on the iodine content of the thyroid gland are shown in Table I and Fig. 1.

TABLE I
Effect of Iodine Intake on Iodine Content of Plasma and Thyroid (Summary Table of Means and Standard Errors)

Group	No. of animals	Iodine content of diet γ per gm.	Daily iodine intake γ	Weight of rats		Weight of thyroid glands mg.	Plasma iodine*		Thyroid iodine	
				At start gm.	At end gm.		Total γ per cent	Protein-bound γ per cent	Total mg. per cent wet weight	Thyroxine mg. per cent wet weight
A	8	0.1 (Approximately)	1-2	207 ± 3.7	284 ± 12.2	26.9 ± 1.7	3.3 ± 0.22	3.1 ± 0.10	21.5 ± 2.8	5.9 ± 0.92
B	8	0.3	3-6		252 ± 6.2	20.0 ± 1.2	3.4 ± 0.07	3.4 ± 0.10	30.7 ± 2.4	9.5 ± 0.58
C	14	1.0	15 ± 0.6	214 ± 4.6	276 ± 8.0	20.0 ± 1.1	5.6 ± 0.27	4.4 ± 0.13	106 ± 6.1	34.7 ± 1.7
D	14	5.0	78 ± 2.7	214 ± 3.6	280 ± 6.9	21.4 ± 1.0	8.8 ± 0.32	5.0 ± 0.14	134 ± 4.9	44.4 ± 1.9
E	14	10	145 ± 7.2	222 ± 5.2	270 ± 9.2	21.0 ± 1.1	13.5 ± 0.88	5.2 ± 0.20	138 ± 2.9	43.0 ± 1.8
F	6	20	310 ± 18	251 ± 5.7	308 ± 9.1	25.7 ± 1.2	21.9 ± 0.74	5.1 ± 0.23	126 ± 8.7	41.0 ± 2.7
G	6	30	440 ± 1.0	255 ± 5.2	299 ± 6.4	27.1 ± 1.3	33.6 ± 2.5		131 ± 19.8	36.5 ± 3.5

* Plasma from two rats was pooled to provide sufficient plasma for the determination of both total and protein-bound iodine on the same plasma sample.

The thyroids of rats that had been maintained on a daily iodine intake of 1 to 2 γ (Group A) were normal in histological appearance and contained from 4.2 to 8.4 mg. per cent of thyroxine iodine (average 5.9) and from 16 to 29 mg. per cent of total iodine (average 21.5).

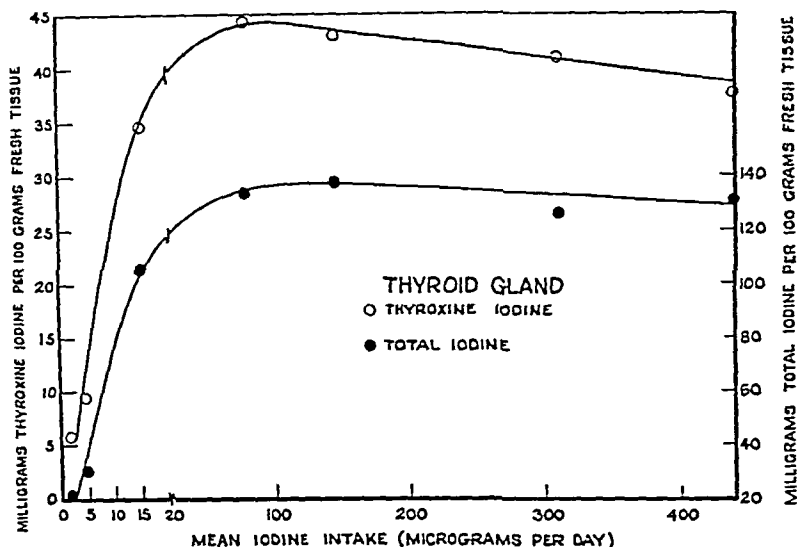


FIG. 1. The relation between iodine intake and total and thyroxine iodine in the thyroid gland.

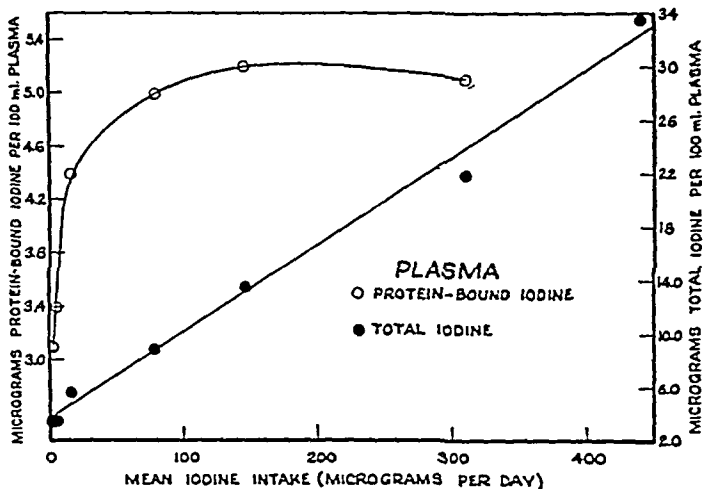


FIG. 2. The relation between iodine intake and total and protein-bound iodine of plasma.

When the daily iodine intake was increased to 3 to 6 γ (Group B) the gland contained 8.3 to 11 mg. per cent of thyroxine iodine (average 9.5) and 27 to 32 mg. per cent of total iodine (average 30.7).

When Diet 2 was supplemented with potassium iodide to provide a mean daily iodine intake of 15 γ and fed to a similar group of rats (Group C), the thyroxine iodine content of the rat thyroids was 28 to 43 mg. per cent (average 34.7) and the total iodine content was 93 to 134 mg. per cent (average 106).

Increasing the mean daily iodine intake to 78 γ (Group D) resulted in a further increase in the thyroxine and total iodine content of the thyroid gland to 37 to 58 mg. per cent (average 44.4) and 115 to 163 mg. per cent (average 134) respectively. Statistical analysis (*t* values) revealed that the mean values for thyroxine and total iodine in this group are significantly higher than those in Group C.

Fig. 1 shows that an increase in the mean daily iodine intake beyond 78 γ produced no further increase in thyroid iodine. In fact, the data suggest an actual decrease in thyroxine iodine at higher iodine intakes.

Plasma Iodine—The effects of the ingestion of increasing amounts of iodine upon the levels of total and protein-bound iodine of plasma are shown in Table I and Fig. 2. In the latter the mean values for total and protein-bound iodine for the various groups have been plotted against the mean iodine intake. The results show that, as the mean iodine intake was increased from 1 to 2 to 78 γ per day, protein-bound iodine of plasma rose from 3.1 ± 0.10 to 5.0 ± 0.14 γ per 100 cc. A further increase in iodine intake failed to produce a significant rise in the level of this plasma constituent. The postabsorptive values for *total iodine*, however, increased almost linearly with iodine intake ($r = 0.96 \pm 0.013$).

DISCUSSION

Remington and Remington maintained several groups of rats on the same basal diet supplemented with iodide to provide daily iodine intakes ranging from 3 to 120 γ (3). They found no consistent increase in the iodine content of the thyroids as the iodine intake was increased. Cole and Curtis reported that rats with an average daily iodine intake of 3 γ per day had the same thyroid iodine content as those with an average intake of 50 γ per day (4). Under the experimental conditions provided here, the level of iodine intake did influence thyroxine storage in the gland. The mean total iodine and thyroxine iodine contents of the thyroids of rats ingesting 3 to 6 γ of iodine per day were 30.7 ± 2.4 and 9.5 ± 0.58 mg. per cent respectively. These glands were found to be normal as regards both size and histological appearance. When the mean daily iodine intake was increased to 78 γ , the mean total and thyroxine iodine

contents rose to 134 ± 4.9 and 41.4 ± 1.9 mg. per cent respectively. The latter values represent the capacity of rat thyroids for the storage of iodine under the present experimental conditions.

The significance of the value representing the upper limit of the iodine-concentrating capacity of rat thyroid is shown by comparing it with the value obtained for plasma iodine. The plasma of rats of Group E (Table I), which ingested daily an average of 145γ of iodine, contained an average of 13.5γ per cent of total iodine. Thus under the conditions that promoted maximal storage, the iodine concentration in thyroid tissue was 10,000 times greater than in plasma.

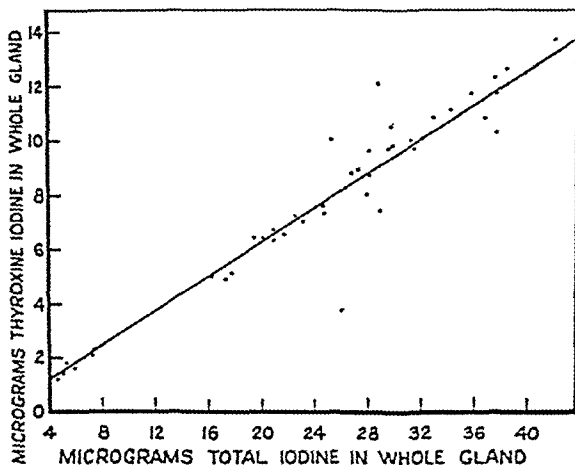


FIG. 3. The relation of thyroxine iodine to total iodine in thyroid glands of rats that received varying amounts of iodine.

Of particular interest is the finding that in the gland the fraction of total iodine present as thyroxine remained relatively constant in rats whose daily iodine intake varied from 1 to 2 to 440γ . This is demonstrated in Fig. 3, in which thyroxine iodine is plotted against total iodine. The mean value for the percentage of total iodine present as thyroxine was 31, with a standard error of ± 0.51 .

Inspection of Figs. 1 and 2 reveals a rough parallelism in the curves depicting the changes in protein-bound iodine of plasma, thyroxine iodine, and total iodine of the thyroid with increasing iodine intake. Each of these three curves rises rapidly at first, reaches its maximum at an intake of about 75γ of iodine per day, and then remains practically level as the iodine intake is further increased. The relation between protein-bound

iodine of plasma and the thyroxine iodine of the thyroid is also expressed by a high linear correlation ($r = 0.84 \pm 0.052$). The above results suggest that in the normal animal the level of protein-bound iodine of plasma is dependent upon the thyroxine content of the thyroid gland and is limited by the gland's capacity to produce thyroxine.

SUMMARY

Uniform groups of rats were maintained on the same basic diet supplemented with different amounts of KI to provide mean daily iodine intakes ranging from 1 to 2 γ to 440 γ . Judged by histological appearance, the thyroid glands obtained from rats of all groups were normal.

1. The amounts of thyroxine and of total iodine in the normal gland were influenced by iodine intake. These amounts increased as the daily mean iodine intake rose from 1 to 2 γ to 78 γ . A further increase was not observed when the mean iodine intake rose beyond 78 γ per day.

2. The *storage capacity* of rat thyroid tissue under the conditions provided here was found to be 130 to 140 mg. per cent for total iodine and 40 to 50 mg. per cent for thyroxine iodine.

3. Despite large differences in the total iodine content of the gland, the percentage of total iodine present as thyroxine remained constant. The mean percentage for forty-four determinations was 31.0 (standard error ± 0.51).

4. The postabsorptive levels of total iodine of plasma were linearly related to the iodine intake ($r = 0.96 \pm 0.013$).

5. An increase in the postabsorptive values for protein-bound iodine of plasma was observed when the mean iodine intake rose from 2 to 78 γ per day. A further increase in iodine intake failed to produce a significant rise in the level of protein-bound iodine of plasma.

6. In the *normal* animal the level of protein-bound iodine of plasma appeared to be dependent upon the thyroxine content of the gland and limited by the gland's capacity to produce thyroxine.

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ON THE ENERGY SOURCE OF THE ACTION POTENTIAL IN THE ELECTRIC ORGAN OF *ELECTROPHORUS ELECTRICUS**

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The energy released by the breakdown of phosphocreatine and the formation of lactic acid appears to be adequate to account for the total electric energy released by the action potential in the electric organ of *Electrophorus electricus* (1). The experiments suggest that energy-rich phosphate bonds are the primary source of energy during recovery, adenosine triphosphate acting as first phosphate donor and phosphocreatine being the "storehouse" for energy-rich phosphate. Thereby it becomes probable that the cellular mechanism yielding the energy for the electric manifestations is basically the same as that known for the mechanical process of muscular contraction.

The electric organs were selected as material for correlating electric and chemical events of the action potential, since both are within the range of accurate measurement, whereas in ordinary nerves considerable difficulties are encountered owing to the small amounts of energy released. The first investigations reported, however, were carried out with very limited material. Only six fish of 160 to 190 cm. length were available. Such large specimens are difficult to handle and are therefore rather inconvenient for experimental analysis. Moreover, the voltage per cm. is considerably lower than in smaller fish (2, 3). It could therefore be expected that small specimens would offer still more favorable material for both the electrical recordings and the chemical analysis. Additional and more precise studies appeared desirable.

Recently a great number of specimens became available and the attempt to correlate chemical and electrical events during the discharge of the electric organ has been resumed. In this paper, further observations on the breakdown of phosphocreatine and lactic acid formation, this time carried out on smaller fish, will be presented.

Methods

Essentially the same methods were used as were described previously (1). Pieces of tissue were cut with the highest possible speed and frozen in liquid

* This work was supported by a grant of the Josiah Macy, Jr., Foundation.

nitrogen. The weight of the tissue used was generally about 200 to 300 mg. 1 ml. of 5 per cent ice-cooled trichloroacetic acid was used per 20 mg. of tissue. The mixture was ground and filtered; an aliquot part of the filtrate was used for lactic acid, another for phosphocreatine determination, with the same technique as described before. Particular care was taken to determine the number of impulses as precisely as possible. The electric scaling circuit described before was so changed that it indicated every thirty-two impulses instead of sixteen. Since sometimes trains of impulses occur at a very high rate, it is difficult to exclude the possibility that occasionally a few trains of impulses are missed. The scaling circuit was therefore replaced by a Potter two decade electronic counter for recording the impulses coupled with a mechanical counter for keeping record of every discharge of the fish. In this way it was assured that the total number of impulses was actually counted. In all experiments of Tables III and IV the new arrangement was used.

The electric recordings, which form the basis of the calculations for the electric energy released, were carried out on the same fish but not simultaneously with the experiments for the chemical analysis. As the electrical characteristics of each individual do not vary when measured on different occasions, it appeared more convenient to carry out the electrical and the chemical observations separately. In order to ascertain that the voltage was about the same as that in the experiments for computing the electric energy, it was always recorded with the aid of a cathode ray oscillograph. These recordings served at the same time as a check that there was no marked decrease in voltage due to fatigue, since this would also affect the chemical reactions. If a marked decrease in voltage occurred, the experiments were discontinued, since in such cases the data are no longer comparable.

Results

Electrical Data—The electrical measurements are reported elsewhere in full detail (4, 5). The data obtained will be described here only as far as they are essential for the interpretation of the chemical figures.

In the previous observations, only that part of the electric energy was actually determined which is released externally (1). In the large specimens used, this energy was calculated to be 8×10^{-6} gm. calorie. Subsequent recordings and several improvements in technique have shown that this figure has to be revised downward to 4×10^{-6} gm. calorie. However, for the problems studied, *viz.* the chemical energy source of the action potential, it is necessary to know the *total* electric energy released per gm. and impulse. The total energy was first estimated to be at least twice as high as that released externally. The determination of the total electric

energy is rather complicated and requires a great number of electrical recordings of various kinds. The investigations of Cox, Coates, and Brown, carried out on the same material as that used for the chemical analysis described in the previous paper as well as in the present, have revealed that the total electric energy per gm. and impulse is close to 24×10^{-6} gm. calorie in the large specimens of 180 cm. average length. This average figure has been obtained by measurements on six fish. The individual values vary between 18 and 35×10^{-6} gm. calorie. In the medium sized fish with which the chemical data described below were obtained, the total energy was found to be 47×10^{-6} gm. calorie, as average of recordings on nine fish, the individual data ranging from 29 to 71×10^{-6} gm. calorie.

Chemical Data—The results of the chemical determinations are summarized in Tables I to IV. It has to be kept in mind that the data obtained are a balance of several reactions going on at any given moment. The phosphocreatine split is continuously being rephosphorylated by the energy released by carbohydrate metabolism, as is known from the studies on muscle (Meyerhof (6)). The oxidative energy is used both for the re-synthesis of carbohydrate and phosphocreatine. Muscle can be isolated and kept in strictly anaerobic condition. In this way, one of the three major sources of energy is excluded and the condition is simplified. A further simplification may be achieved in the case of muscle by inhibition with monoiodoacetic acid, blocking the energy of carbohydrate as was shown by Lundsgaard. Then phosphocreatine becomes the only source providing energy for the contraction. No such simplification separating the different reactions has yet been devised for the electric organ of the living animal. However, both the basic respiration and the increase in oxygen uptake following the action potential are probably small, as may be concluded from all measurements on nerve respiration and from the low succinic oxidase content of electric tissue (2). It appears probable therefore that during the short period of the experiments the correction which has to be made owing to increased respiration is small, only a few per cent of the two reactions measured. The amount found is therefore probably close to the total energy released anaerobically.

Since it is known from the observations on muscle that, under certain conditions, part of the lactic acid formation may be delayed, i.e. is going on for some time after the relaxation of the muscle, it is possible to assume that a similar delayed lactic acid formation may occur following the discharge. In that case, if the tissue is cut and frozen immediately after the period of discharges, the lactic acid formed following the discharge may not be the maximal amount. It therefore appeared desirable to allow for a "recovery period" during which the delayed lactic acid formation would occur. Tables I and II summarize the observations with such a recovery

TABLE I

Energy Released per Gm. and Impulse by Breakdown of Phosphocreatine during Action Potential of Electric Organ of Electrophorus electricus

Medium sized fish. The voltage given is that recorded per 10 cm. with an external resistance of 200 ohms, that in parentheses with an open circuit. After about 1300 to 1500 impulses, stimulation was avoided and a recovery period of 3 to 4 minutes was permitted before a piece of tissue was cut and frozen for chemical analysis. The number of discharges at the end of the recovery period is the total number between the first and second cuts.

Experiment No.	Length of fish	Voltage	Period		No. of discharges	Initial P_2O_5	Phosphocreatine split	Gm. calories $\times 10^{-4}$ per gm. and impulse
			Stimulation	Recovery				
	cm.		min.	min.		mg. per gm.	mg. P_2O_5 per gm.	
1	89.0	61 (121)	4.0		1408	1.20	0.49	44.7
				3.5	1696			
2	78.0	78 (151)	3.5		1440	0.88	0.71	60.2
				4.5	1824			
3	100.5	77 (135)	3.5		1440	0.99	0.79	76.5
				4.0	1600			
4	93.5	70 (118)	3.0		1472	1.38	0.46	43.7
				3.0	1632			
5	112.5	54 (102)	2.5		1280	1.47	0.35	41.5
				4.0	1312			
6	111.0	58 (106)	3.0		1280	1.71	0.28	34.0
				3.0	1280			
7	104.0	76 (138)	3.0		1280	1.445	0.75	84.6
				3.0	1376			
8	103.0	62 (118)	3.0		1280	1.83	0.45	54.5
				3.0	1280			
9	95.0	82 (154)	4.5		1280	1.285	0.595	62.8
				3.0	1472			
10	110.0	52 (104)	3.5		1088	1.395	0.595	85.0
				3.0	1088			
11	143.0	52 (83)	3.0		1568	0.945	0.46	45.5
				3.0	1568			
12	112.0	60 (104)	3.0		1546	1.56	0.65	65.0
				3.0	1546			
13	107.0	60 (114)	3.5		1568	1.57	0.67	56.0
				3.0	1856			
14	105.0	52 (106)	3.0		1600	1.715	0.575	55.6
				3.0	1600			
15	90.0	64 (110)	3.0		1536	1.635	0.395	39.0
				3.0	1568			
Average. . . .	103.6							56.6

period. After 1200 to 1500 discharges within a period of 3 to 4 minutes, the fish was kept as quiet as possible and any further stimulation was avoided. This was in most cases successful. Practically none or very few spontaneous discharges occurred during the recovery period. In some cases there were still a few spontaneous discharges but their number was insignificant when compared with the total.

TABLE II

Energy Released per Gm. and Impulse by Lactic Acid Formation

The experiments are the same as in Table I; the determination of lactic acid has been carried out with an aliquot part of the filtrate used for phosphocreatine determination. The last column indicates the energy released by the two processes (phosphocreatine breakdown and lactic acid formation).

Experiment No.	Initial lactic acid	Lactic acid formed	Gm. calories $\times 10^{-4}$ per gm. and impulse	Gm. calories $\times 10^{-4}$ per gm. and impulse (sum)
	<i>mg. per gm.</i>	<i>mg. per gm.</i>		
1	0.140	0.321	46.1	90.8
2	0.177	0.283	37.9	98.1
3	0.396	0.337	51.5	128.0
4	0.469	0.236	33.7	77.4
5	0.383	0.367	69.5	111.0
6	0.347	0.308	58.5	92.5
7	0.372	0.418	74.2	158.8
8	0.410	0.345	57.3	111.8
9	0.540	0.490	81.0	143.8
10	0.442	0.428	96.1	181.1
11	0.278	0.476	74.0	119.5
12	0.331	0.532	84.0	149.0
13	0.278	0.462	60.5	116.5
14	0.195	0.432	66.0	121.6
15	0.463	0.521	81.0	120.0
Average.....			64.8	121.3

In Tables III and IV are given data obtained in experiments in which no recovery period was permitted and in which the tissue cut was performed directly at the end of the stimulation period. If these data are compared with those in Tables I and II, it appears that there is no significant difference, the average figures being 121.3 and 126.4×10^{-4} gm. calorie respectively. In view of the individual variations, such a difference is small and, moreover, in a direction opposite to the expected, the lower average figure being that of the experiments with recovery.

In this case a fundamental difference between nerve and muscle function has to be kept in mind. The duration of the action potential is of the

order of 1 millisecond, that of muscular contraction several hundred times higher. The energy required for contraction is many thousand times higher than that for the passage of the impulse. For both reasons, a longer period of lactic acid formation may be expected in the case of muscle activity.

TABLE III

Breakdown of Phosphocreatine As in Table I, but with Tissue Cut and Frozen Immediately at End of Stimulation Period

Experiment No.	Length of fish	Voltage*	Stimulation period	No. of discharges	Initial phosphocreatine	Phosphocreatine split	Gm. calories $\times 10^{-4}$ per gm. and impulse
	cm.		min.		mg. P_2O_5 per gm.	mg. P_2O_5 per gm.	
16	98	45 (92)	4.5	1500	1.505	0.375	38.7
17	96	49 (82)	2.0	1600	1.52	0.56	54.5
18	95	34 (77)	2.0	1600	1.605	0.565	54.7
19	102.5	70 (110)	4.5	1500	1.26	0.43	44.3
20	108	73 (100)	2.0	1900	1.31	0.55	44.7
21	94	53 (90)	2.0	1600	1.605	0.565	54.7
Average.....	99						48.6

* Voltage recorded per 10 cm. with an external resistance of 200 ohms; that in parentheses, with an open circuit.

TABLE IV

Lactic Acid Formation of Experiments Reported in Table III (without Recovery Period)

The last column indicates the energy released by both the phosphocreatine breakdown and lactic acid formation.

Experiment No.	Initial lactic acid	Lactic acid formed	Gm. calories $\times 10^{-4}$ per gm. and impulse	Gm. calories $\times 10^{-4}$ per gm. and impulse (sum)
	mg. per gm.	mg. per gm.		
16	0.296	0.694	113.0	151.7
17	0.143	0.447	68.0	122.5
18	0.227	0.418	63.5	118.2
19	0.224	0.604	98.0	142.3
20	0.293	0.479	61.5	106.2
21	0.218	0.410	62.5	117.2
Average.....			77.8	126.4

The observations suggest that the total lactic acid formed as a result of the discharges has actually been determined under the conditions used.

The calculations of the energy provided by the breakdown of phosphocreatine were previously based on the assumption of 10,000 gm. calories

per mole of phosphocreatine (1). It appears, however, more correct to assume 11,000 gm. calories in view of different recent estimations (Meyerhof (7)). The value is about the same for all energy-rich phosphate bonds. The figures given in this paper are calculated on this basis. The formation of 1 mole of lactic acid yields about 16,000 gm. calories without neutralization heat. In the previous calculation this figure was used (1). Since it is known that 2 moles of energy-rich phosphate bonds may be created per mole of lactic acid, 22,000 gm. calories per mole of lactic acid seem to be, according to Meyerhof, a more correct assumption (8). This higher figure has been used for the calculation of the figures presented.

In Table V are summarized the electrical and chemical data obtained so far. Two facts emerge from these figures: (1) in the medium sized fish, the total of the electric energy and the chemical energy measured is

TABLE V

Relationship between Electric Energy Released by Action Potential and Chemical Energy Released by Breakdown of Phosphocreatine and Formation of Lactic Acid

Average values. The figures in parentheses indicate the number of experiments on which the average values are based.

Length of fish cm	Energy released per gm. and impulse, gm. calories $\times 10^{-6}$				Electrical energy in per cent of chemical
	Electrical	Phosphocreatine	Lactic acid	Combined	
103	47 (9)	54.5 (21)	63.5 (21)	123.0 (21)	38
180	24 (6)	35.7 (15)	23.1 (7)	53.4 (7)	45

about twice as high as in the larger specimens; (2) the energy of phosphocreatine breakdown and lactic acid formation exclusive of the oxidative energy seems to be more than twice as high as the electrical energy. In still smaller fish, of 67 cm. average length, the total electric energy released per gm. and impulse is markedly higher. In these fish 88×10^{-6} gm. calorie per gm. and impulse was found as the mean value of eight experiments, the individual values varying between 55 and 118×10^{-6} gm. calorie. The chemical energy also appears to be markedly higher, but insufficient measurements were made and more experiments are necessary for obtaining a reliable picture.

DISCUSSION

The surface changes occurring during an electric discharge involve loss of energy. The reversal of this process can conceivably be effected only by the free energy of chemical reactions. The observations presented support and amplify the evidence offered previously that phosphate bonds are

the source of free energy for the recovery, by which the restitution of the resting condition is achieved, following the action potential.

In the preceding studies, the chemical energy measured was correlated only with the energy released externally by the discharge, since insufficient data were available for computing the total energy released. Even now the determination of the total electric energy is subject to a number of uncertainties. The most important is the question of whether or not the electromotive force remains unchanged during the whole period of the discharge. All measurements indicate that the resistance undergoes considerable changes during the discharge but the electromotive force remains essentially constant throughout its duration. However, it cannot be excluded that, during a short period of the phase of rising voltage, variation of the electromotive force does occur. For the calculation of the electric energy released, constant electromotive force has been assumed. Fortunately for this calculation, the uncertain phase is of extremely brief duration compared with the rest of the discharge. It therefore appears safe to assume that the order of magnitude computed would not be affected materially if a change in the electromotive force does occur in the early part of the initial phase.

On the basis of the present data, the electric energy is about 40 per cent of the chemical energy released by the two anaerobic processes measured. Assuming that oxidation would provide about the same amount of energy for the reversal of the two anaerobic processes, the electric energy would be close to 20 per cent of the total chemical energy. This would be a reasonable efficiency.

It would be desirable to complement these data by a study of the heat production, since only in this way could the total energy change be computed. Such measurements are being prepared.

The question as to whether or not the discharge of the electric organ may be compared with the ordinary nerve action potential has been frequently discussed (9). According to Hill, the fundamental physicochemical mechanism is almost certainly the same (10). The electric organs are an accumulation of motor end plates and their action potential may be best compared to the end plate potential studied recently by Eccles and his associates (11). There is no reason to assume that axon and end plate potential differ basically except in a quantitative respect. In fact, the data available so far are consistent with the assumption of a similar physicochemical mechanism (12).

SUMMARY

Observations are presented supporting and amplifying the previous evidence that the breakdown of phosphocreatine provides the chemical

energy for the restitution of the resting condition following the electric discharge of the electric organ of *Electrophorus electricus*. Electric recordings and chemical determinations carried out on a greater amount of more favorable material than available before have revealed the following facts:

1. In medium sized fish of average length of 103.6 cm., the breakdown of phosphocreatine yields 54.5×10^{-3} gm. calorie per gm. and impulse. The formation of lactic acid yields 68.5×10^{-3} gm. calorie per gm. and impulse. Both figures are mean values of twenty-one experiments. The chemical energy released by these two reactions is consequently 123.0×10^{-3} gm. calorie per gm. and impulse. The total electric energy released in the same specimens is 47×10^{-3} gm. calorie per gm. and impulse (average of nine experiments).

2. No difference is found in lactic acid formed whether the determination is made directly at the end of the stimulation period or at the end of a recovery period.

3. The chemical energy in the medium sized specimens is about twice as high as in large fish of 180 cm. average length in which the two chemical reactions measured were found to yield 53.4×10^{-3} gm. calorie per gm. and impulse (mean value of seven experiments). This is in good agreement with the equally smaller electric energy released in these specimens which is 24×10^{-3} gm. calorie per gm. and impulse (average of six experiments).

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THE ISOLATION OF PECTIC SUBSTANCES FROM SOFTWOODS*

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Branfoot (1) has summarized the early work on pectic substances. Bonner (2), Norman (3), and Hirst and Jones (4) have reviewed later work on the structure of these substances.

Pectic materials that approximate the composition and have some of the properties of pectic acid are widely distributed but in small amounts in mature hardwoods. In 1925 O'Dwyer (5) isolated such a material from beechwood, and in 1931 Preece (6) reported 0.4 per cent of pectin in boxwood. Since then pectic materials have been isolated from mature wood of black locust (7), lemon, mesquite, and white pine (8), and from cottonwood (9).

In mature woods pectic materials are mixed or combined with large amounts of cellulose, lignin, hemicellulose, and often with other carbohydrate materials. Although they have been isolated from mature hardwoods and from one softwood, it is far more difficult to isolate them in appreciable amounts from softwoods. This possibly is due to the greater solubility of the hemicelluloses of softwoods than of hardwoods and to the greater amount of other soluble carbohydrates that dissolve with the pectic materials in the extraction of softwoods. No satisfactory procedure for isolating these materials from mature woods and for separating them from the accompanying substances is known. The methods now used are not quantitative. They require large amounts of wood if sufficient pectic material is to be obtained for complete purification. Undoubtedly the pectic materials so far isolated from wood have undergone considerable hydrolysis in the process of isolation and purification. As a result, pure pectic materials from mature woods have not been studied sufficiently for their properties and composition to be accurately determined.

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The purest of these materials that have been isolated from mature wood have the following properties: In a 2 per cent solution of ammonium hydroxide they show $[\alpha]_D$ of approximately $+235^\circ$. This is somewhat less than the $[\alpha]_D$ of pectic acid that has been prepared from citrus pectin through the calcium salt (8). The free acids yield from 19.5 to 22 per cent carbon dioxide by the method of Lefèvre and Tollens (10) and approximately 19.5 per cent furfural. The calcium salts contain from 7.5 to 9.5 per cent calcium. They are more soluble and gel to a less extent when precipitated with calcium chloride than does calcium pectate which has been prepared from citrus pectin. They also differ in their solubility, probably because of variation in their degree of hydrolysis. The presence of galacturonic acid can be readily established by conversion to mucic acid by the method of Heidelberger and Goebel (11).

The present investigation was undertaken to determine how widely pectic materials occur in mature softwoods and to improve the method of isolating and purifying them.

EXPERIMENTAL

Establishing Presence of Pectic Substances in Softwoods—Several attempts were made to isolate pectic material in appreciable amounts from Douglas fir and western hemlock by extracting 3 kilo lots of the powdered woods in succession with hot water, hot 0.05 N hydrochloric acid, and 5 per cent ammonium hydroxide. In these investigations the extracts were concentrated and decolorized by bromine, as described later, but they were separated into fractions by precipitation with alcohol. Calcium chloride was used only to a very limited extent in this process. In each investigation some material was obtained that showed $[\alpha]_D^{25}$ as high as $+80^\circ$ and gave as high as 10 per cent of carbon dioxide. However, the amounts of pectic substances obtained were too small for thorough purification and the results were not conclusive.

Western red cedar, when extracted and decolorized in the same manner, gave, before fractionation with alcohol, 1.23, 0.35, and 0.7 per cent of the hot water, hot 0.05 N hydrochloric acid, and 5 per cent ammonium hydroxide extracts. In place of simple fractionation with alcohol, the pectic material in these extracts was converted to calcium pectate and back to the free pectic acid twice. They then showed $[\alpha]_D^{25} = +60^\circ$, $+50^\circ$, and $+206^\circ$. On analysis they gave 5.93, 13.4, and 15.3 per cent carbon dioxide and 17.2, 16.25, and 23.6 per cent furfural. After reconversion of the ammonium hydroxide extract to calcium pectate and back to the free pectic acid two more times, it showed $[\alpha]_D^{25} = +247^\circ$ and gave 18.1 per cent carbon dioxide and 21.1 per cent furfural. When it was tested by the method of Heidelberger and Goebel it gave mucic acid, melting at 218° .

The results obtained with western red cedar indicated that, in the isolation of pectic materials from softwoods, purification of the extracts by repeated conversion to calcium pectate and back to pectic acid was far superior to fractionation by alcohol. As a result the procedure described below was followed in a further study of Douglas fir and western hemlock.

Isolation of Pectic Material from Wood of Douglas Fir and Western Hemlock—3 kilos of the powdered woods were repeatedly extracted under a reflux with a benzene-alcohol mixture until the extracts were no longer deeply colored.

The lipide-free woods were then extracted twice in baths of boiling water, each time for 6 hours, with 9 times their weight of water. The extracts were concentrated *in vacuo* at 75° to 500 cc. To the concentrates were added 20 gm. of calcium chloride dissolved in 30 cc. of water, followed by 8 volumes of alcohol. The precipitates were isolated and washed with alcohol and ether.

The woods were next heated with 8 times their weight of 0.05 N hydrochloric acid in baths of boiling water for 5 hours and filtered. The filtrates were neutralized with a slight excess of ammonia and concentrated. The concentrates were made slightly basic with ammonia and then slightly acid with acetic acid and mixed with calcium chloride solution and alcohol. The precipitates were isolated as described above. The woods were finally extracted twice, at room temperature, with 5 per cent ammonium hydroxide, each time for 6 days. The filtrates from each wood were concentrated and the concentrates were treated with acetic acid, calcium chloride, and alcohol, as in the case of the hydrochloric acid extract.

Isolation of Pectic Material from Holocelluloses of Douglas Fir and Western Hemlock—1 kilo of the coarsely ground sawdust in amounts of 100 to 300 gm. was converted to holocellulose, as described by Wise (12). Exhaustive treatment with sodium chlorite in the preparation of holocellulose causes loss of carbohydrate material and should be avoided. The holocellulose used here still contained as much as 2 per cent lignin. After thorough washing with acetone, the holocellulose was extracted for 3 days at room temperature with approximately 20 times its weight of a 2.5 per cent solution of ammonium hydroxide. The extract was treated exactly as those in the preceding section. One extraction with a 2.5 per cent ammonium hydroxide dissolves most of the pectic material. Even this extraction dissolves considerable hemicellulose and other carbohydrate material with the pectic material.

Removal of Lignin from Extracts—To remove lignin and coloring matter from the extracts of wood or holocellulose, it was rubbed with a cold 1 per cent solution of hydrochloric acid and shaken with small amounts of liquid bromine. After an hour 8 volumes of alcohol were added. This reacts

with excess bromine, dissolves the calcium chloride and chlorinated lignin, and precipitates the pectic materials, hemicelluloses, arabogalactan, and related substances. The precipitate was filtered and washed with alcohol and ether. It was then dissolved in a 2 per cent solution of ammonium or sodium hydroxide and the solution filtered. The filtrate was made slightly acid with cold dilute hydrochloric acid and shaken with liquid bromine. After the dark color had disappeared and bromine was no longer absorbed, 8 volumes of alcohol were added and the purified material was isolated. This treatment with bromine was repeated until the material was white. In this procedure, hot strongly acid or alkaline solutions were avoided in order to minimize hydrolysis of the materials (13).

Purification of Pectic Materials Extracted from Softwoods or from Hemicelluloses of Softwoods—The purification of pectic materials extracted from hardwoods has been briefly described in previous publications (7, 8). The separation of the pectic materials from the other carbohydrates present in the extracts depends on the difficult solubility of calcium pectate in water and 25 per cent alcohol. In the separation, the pectic materials are repeatedly converted into difficultly soluble calcium pectate and back to free pectic acid. The exact procedure to be followed depends on three factors: (1) what other carbohydrates are present in the extracts with the pectic materials; (2) how much the pectic material has been hydrolyzed during its isolation; and (3) whether it is present as a calcium salt or as free pectic acid. If the pectic material is present as a calcium salt, it must be converted to the free acid by treatment with a 1 per cent solution of hydrochloric acid and 8 volumes of alcohol before it can be dissolved in the ammonium or sodium hydroxide. If a hemicellulose is present with the pectic material, a 2 per cent solution of ammonium hydroxide may not dissolve the extract and a 2 per cent solution of sodium hydroxide must be used. Also, if a hemicellulose is present when the solution is acidified, a precipitate may form. This should be centrifuged out and its $[\alpha]_D^{25}$ determined. If the pectic acid has not been appreciably hydrolyzed during isolation, it will precipitate as calcium pectate. Usually, however, considerable hydrolysis has occurred and 0.5 volume of alcohol must be added to precipitate all of the calcium pectate.

In general the decolorized extract is dissolved in a 2 per cent solution of ammonium hydroxide and filtered. The filtrate is made slightly acid with acetic acid, and 20 gm. of calcium chloride in 30 cc. of water are added, followed by 0.5 volume of alcohol. This precipitates any pectic acid present, together with some of the other carbohydrates. The precipitate is centrifuged out and washed with 25 per cent alcohol. The solution contains most of the other carbohydrates which are precipitated by addition of 8 volumes of alcohol. The calcium pectate is reconverted to pectic acid.

By numerous repetitions of this procedure the extracts are separated into free pectic acid, arabogalactan (if any is present), and hemicellulose. Each step in the procedure should be followed with the polariscope. The pectic acid rotates strongly dextro, the arabogalactan rotates approximately $+12^\circ$, and the hemicellulose rotates strongly levo.

Results Obtained with Western Hemlock and Douglas Fir—Western hemlock wood, treated as described above, gave a total of 0.17 per cent of purified pectic acid, whereas the holocellulose gave 0.55 per cent of the weight of the wood as purified calcium pectate. The pectic acid from the wood showed $[\alpha]_D^{25} = +175^\circ$ and on analysis gave 16.03 per cent carbon dioxide and 15.34 per cent furfural. The calcium pectate from the holocellulose gave 7.35 per cent calcium, 16.25 per cent carbon dioxide, and 20.35 per cent furfural. When this calcium pectate was converted to free pectic acid, the acid showed $[\alpha]_D^{25} = +216.3^\circ$ and gave 19.7 per cent carbon dioxide. When the pectic acid was treated by the method of Heidelberger and Goebel, it gave mucic acid, melting at $217-218^\circ$.

The extracts of Douglas fir before purification amounted to 5.1 per cent of the wood. The 2.5 per cent ammonium hydroxide extract of the holocellulose of Douglas fir amounted to 4.25 per cent of the wood used. The loss during decolorization and purification of the pectic material was so large that no accurate estimate of the amount of pure pectic acid obtained could be made. It was between 0.1 and 0.2 per cent. The purified pectic acid from the various fractions showed $[\alpha]_D^{25}$ from $+75^\circ$ to $+115^\circ$, and gave from 5.2 to 19.4 per cent carbon dioxide. These purified extracts were combined and twice converted to calcium pectate and back to pectic acid. This showed $[\alpha]_D^{25} = +234.8^\circ$ and gave 20.05 per cent carbon dioxide and 19.1 per cent furfural. When treated by the method of Heidelberger and Goebel, it gave mucic acid, melting at 218° .

The results obtained with western red cedar, Douglas fir, and western hemlock establish conclusively the presence of pectic substances in these three softwoods.

Isolation of Pectic Materials from Smaller Amounts of Softwoods—Smaller amounts than previously used of the following five softwoods were converted to holocelluloses by Dr. L. E. Wise: Douglas fir (*Pseudotsuga taxifolia*); western hemlock (*Tsuga heterophylla*); western red cedar (*Thuja plicata*); loblolly pine (*Pinus taeda*); and black spruce (*Picea mariana*). These holocelluloses were extracted by the author for 3 days with 2.5 per cent ammonium hydroxide and the extracts were isolated as previously described. The results are summarized in Table I.

The first ammonium hydroxide extracts of Table I were then decolorized by bromine. The resulting products are the decolorized extracts of Columns 2 and 3, Table II. These decolorized extracts of Table II were then

separated by repeated treatment with calcium chloride into an insoluble calcium pectate and a soluble hemicellulose (Fractions A and C, Table II). The analytical determinations made on these fractions are summarized in Table II.

Fractions A of Douglas fir, western hemlock, and loblolly pine, whose analyses are given in Table II, were purified further by conversion to cal-

TABLE I

Yields of 2.5 Per Cent Ammonium Hydroxide Extracts of Holocelluloses from Five Woods

Wood No. (1)	Wood used (2)	Holocellulose yield (3)	Ammonium hydroxide	
			1st extract (4)	2nd extract (5)
	gm.	per cent	per cent	per cent
1	267	72.1	14.5	1.6
2	186.5	74.1	14.5	1.4
3	187	63.6	14.3	0.5
4	368	70.4	13.9	0.7
5-I	142	57.7	7.1	0.5
5-II	177.4	74.2	15.1	

TABLE II

Summary of Analysis of Pectic Materials from Holocelluloses of Five Softwoods

Wood No. (1)	Decolorized extract		Fraction A (4)	Pectic acid, Fraction A				Hemicellulose, Fraction C		
		$[\alpha]_D$		$[\alpha]_D$	Carbon dioxide	Furfural	Pectic acid in Fraction A (8)	Pectic acid in wood (9)		$[\alpha]_D$
	gm.	degrees	gm.	degrees	per cent	per cent	gm.	per cent	gm.	degrees
1	8		1.9	+144	14.23	19.7	1.14	0.43	3	-25.1
2	12	0	1.14	+183			0.85	0.45	8	-17.5
3	7	-23.4	0.35	+112.4	12.96	22.9	0.20	0.11	5	-28.1
4	16	-10.3	2.5	+112.5			1.25	0.34	10	-25.2
5-I	2.7		0.4	+83.8			0.20	0.14	2.4	-32.4
5-II	3.7		1.7	+166			1.13	0.64	16	-32.7

cium pectate and back to free pectic acid. The resulting materials showed, respectively, $[\alpha]_D^{25} = +200^\circ$, $+212^\circ$, and $+205^\circ$. They gave 18.6, 19.7, and 19.6 per cent carbon dioxide and 19.7, 20.5, and 22.8 per cent furfural.

These results show that smaller amounts of softwoods than those previously used are sufficient for the isolation of pectic materials if they are first converted to holocelluloses.

Explanation of Table I—The yields of ammonium hydroxide extract in Column 4 are in percentage of the holocellulose. The woods used were (1)

Douglas fir; (2) western hemlock; (3) western red cedar; (4) loblolly pine; (5-I) black spruce I; (5-II) black spruce II. Wood 5-I was treated too long with sodium chlorite. This removed all of the lignin, and some of the carbohydrate material was lost. Wood 5-II was treated for a shorter length of time. Table I shows that one extraction with 2.5 per cent ammonium hydroxide removes approximately the same percentage of extract from all of the woods. The second extract was small. Specific rotations which were made on the second extracts indicated that they were hemicelluloses.

Explanation of Table II—The woods are the same as those in Table I. The decolorized extracts were prepared from the first ammonium hydroxide extracts of Table I by bromination. Fractions A and C were prepared from the decolorized extracts by repeated treatment with calcium chloride. Fraction A is from the calcium pectate precipitate. Fraction C is from the filtrate from Fraction A. The gm. of pectic acid in Fraction A, Column 8, were calculated from the gm. and $[\alpha]_D^{25}$ of Fraction A, Columns 4 and 5, on the assumption that pure pectic acid would show $[\alpha]_D^{25}$ of approximately $+250^\circ$.

Table II shows that the 2.5 per cent ammonium hydroxide extracts of the holocelluloses of these five softwoods were separated into a pectic acid fraction and a hemicellulose fraction and that pectic materials are present in all five of these woods.

SUMMARY

1. The presence of pectic substances has been established in five important mature softwoods. Since their presence has previously been established in some mature hardwoods, pectic materials appear to be normal constituents in many mature woods.

2. Although the methods used in isolating pectic materials from wood are not quantitative, they may be present up to 0.5 per cent in softwoods.

3. These materials approximate the composition and have some of the properties of pectic acid. Changes in the solubility and losses during purification can best be explained on the assumption that some hydrolysis occurs during isolation and purification of the pectic substances.

4. An improved method has been devised for isolating pectic materials from softwoods by converting the wood to holocellulose and extracting this with a 2.5 per cent solution of ammonium hydroxide.

5. An improvement has been made in the method of separating pectic materials from lignin, hemicellulose, and other carbohydrates that are coextracted from wood.

6. Failure to remove pectic materials from hemicelluloses will lead to considerable error in the analyses of the latter because of the high uronic acid content and high specific rotation of the pectic materials.

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ISOLATION AND CHARACTERIZATION OF THE T₂ BACTERIOPHAGE OF *ESCHERICHIA COLI**

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PLATES 1 TO 3

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A number of viruses responsible for disease in plants and animals have been purified in recent years and much has been learned of their chemical, physical, and, following the introduction of the electron microscope, morphological characters. In contrast, the bacterial viruses have been given relatively little attention in the application of the methods of purification and study employed for other viruses. Moreover, much of the significance of the results thus far obtained is obscured by the use of different bacteriophages in the various investigations. Schlesinger (1-3); combining ultrafiltration and centrifugation, purified a bacteriophage of *Bacillus coli*¹ and found it to be constituted of protein and an ether-soluble fraction. The material contained 3.7 per cent phosphorus, which was considered (3,4) to be bound in nucleic acid. Tests for carbohydrate were only weakly positive. Using chemical methods, Northrop (5) isolated a "macromolecular nucleoprotein" from lysates of cultures of staphylococcus. The material, which possessed the biological properties of the phage, gave sharp boundaries in sedimentation diagrams in the ultracentrifuge (6), but unsedimentable material was generally present. Similar chemical findings were encountered by Pollard (7) in analyses of a *Bacillus coli* bacteriophage purified by McIntosh and Selbie (8). Kalmanson and Bronfenbrenner (9), studying the P. C. phage of *Escherichia coli*, found a low phosphorus content (0.07 per cent), and considered that the active agent was protein rather than nucleoprotein. Lipide and carbohydrate were not determined.

Electron micrographic studies of bacteriophage lysates (10-13) of various bacteria have greatly advanced the status of the problem of the mor-

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¹ The terminology *Bacillus coli* is used wherever it was employed by the authors to whom reference is made.

phology of these bacterial viruses. There still remain, however, many questions concerning the physical and chemical characters of the agents, and, for this reason, further experiments have been undertaken on the purification and study of bacteriophage. The agent chosen was the T₁ phage of *Escherichia coli*, which was the same as that previously investigated by Kalmanson and Bronfenbrenner (9). This agent has been concentrated and at least partially purified by Anderson (14). The electron micrographic appearance of the purified material obtained in the present work has already been described briefly (15). The present paper is devoted chiefly to a description of the technique of purification and to the general chemical² and physical characters of the agent.

Materials and Methods

The *Escherichia coli* bacteriophage, P. C., of Kalmanson and Bronfenbrenner (9) has been designated as γ by Delbrück and Luria (16) and as T₂ by Anderson (14) and others. The strain of bacteriophage, obtained through the generosity of Dr. Max Delbrück, Vanderbilt University, had been cultured on the host in synthetic medium. From this preparation, stock phage was prepared in nutrient broth, as described below. A volume of 500 ml. was passed through a 5 × 1 inch Berkefeld N filter, followed by 100 ml. of distilled water. The suspension, stored at 2–8°, maintained its potency of about 10¹⁰ plaque-forming particles per ml. throughout the 6 months duration of these experiments.

The host organism, *Escherichia coli*, strain B (9, 16), also obtained from Dr. Delbrück, was maintained on 1 per cent nutrient agar slants with transfers at intervals of 1 day. For each experiment the organism was transferred to broth.

Purification was carried out in parallel on phage cultured on bacteria grown in (1) broth and (2) synthetic medium. The media and methods of culture were essentially those described by Delbrück (17). The broth was Difco nutrient broth, pH 6.5 to 7.0, to which NaCl was added to 0.5 per cent concentration. The synthetic medium consisted of Na₂HPO₄ 0.6, KH₂PO₄ 0.3, MgSO₄ 0.02, NaCl 0.05, NH₄Cl 0.1, and glucose (c.p. grade) 0.4 per cent. The glucose was autoclaved separately in 4 per cent solution and added aseptically to the autoclaved salt base. The pH of the final medium was 6.9 to 7.1. The nutrient agar for carrying the stock culture, as described above, and for titration of the phage, was Bacto-standardized agar in 1 per cent concentration in nutrient broth.

In the production of phage for concentration and purification, batches

² A portion of the work was described by one of the authors (A. R. T) before the meeting of the American Chemical Society at Atlantic City, New Jersey, April 12, 1946.

of 7.5 to 15 liters, distributed in 1500 ml. volumes in 2 liter Florence flasks proved convenient. Seed inoculum of bacteria for the large volumes was prepared in 50 ml. of the respective media in 125 ml. Erlenmeyer flasks, inoculated with 0.1 ml. of an 18 to 24 hour broth culture of *Escherichia coli*, and incubated for 18 hours at 37°. The broth inoculum, after incubation, contained about 2.0×10^8 viable cells per ml. Stock phage containing 10^{10} lytic units per ml. was added in the ratio of 1 phage particle to 400 to 700 bacteria, and the flask was shaken and allowed to stand for 5 to 7 minutes. To each 1500 ml. volume of medium, previously warmed to 37°, there were added 30 ml. of the phage-bacteria suspension. The flasks were incubated for 8 hours at 37° with vigorous mixing, manually, every 15 minutes. (The 500 ml. of stock inoculum described above were prepared from a broth culture at this stage.) The individual batches of nutrient broth cultures were pooled immediately and stored in the ice box for 7 to 14 days before processing. The synthetic medium cultures were left overnight in the incubator, without further agitation, before pooling. The nutrient broth cultures usually were completely lysed at the end of 8 hours; synthetic medium cultures were not, even after 24 hours incubation.

Titration of lytic units was accomplished essentially as described by Ellis and Delbrück (18). Dilutions to be tested were made in broth in successive 10-fold increments, and 0.5 ml. quantities of those in the appropriate range were mixed, each with 2.0 ml. volumes of 18 hour-old broth cultures of the bacterium. After 5 minutes, 0.1 ml. volumes of the mixtures were spread on agar plates, three for each dilution tested. The plaques were counted after incubation of the plates at 37° for 16 to 24 hours.

The purification and analytical procedures employed were similar to those used in the work with the papilloma (19) and influenza viruses (20, 21). The modified laboratory model air-driven Sharples supercentrifuge (20) was used for the initial concentration of the phage from the lysates. For further purification, the vacuum ultracentrifuge (22) with a capacity of 120 ml. per run was used. Sedimenting boundaries of the concentrated materials were photographed in the air-driven analytical ultracentrifuge (23) by means of ultraviolet light of 2400 to 2800 Å, by the absorption method of Svedberg. The electron microscope was the RCA type B instrument. All micrographs were made with 55 kilovolt electrons.

Isolation of Bacteriophage from Lysed Nutrient Broth Cultures

While the phage was readily concentrated by the centrifugation of freshly lysed nutrient broth cultures of 8 hours incubation, purification at this stage was very difficult. The fresh lysates contained much mucoid material, and the phage particles were apparently trapped in or coated with slimy substance. Filtration of such lysates or preliminary low speed clarification runs in the Sharples supercentrifuge for removal of residual

bacteria resulted in the loss of 60 to 80 per cent of the phage activity. Much of this phage, sedimented in the low speed centrifugation, could be recovered by redispersing the sediment in saline solution and filtering with Celite, but this practice was not efficient, since subsequent concentrates were always heavily contaminated with unsedimentable protein or other ultraviolet light-absorbing material.

Contrary to the findings with the fresh lysates, however, it was observed that the troublesome effects of the mucoid material could be avoided by allowing the lysates to stand in the refrigerator for a time before applying the purification procedures. During this period, a granular or flocculent precipitate sedimented in progressively increasing amounts in association with a diminution of the slimy material. Studies on lysates at various intervals showed that the most satisfactory results, with respect both to yield and to the elimination of the mucoid material, were obtained when the lysates were kept in the refrigerator for 7 to 14 days before purification was begun.

Elimination from the lysates of bacteria and bacterial debris, as well as most of the flocculated mucoid material, after the interval of 7 to 14 days was accomplished in the earlier experiments by preliminary low speed spinning in the Sharples centrifuge. The pooled lysate (crude lysate) was run into the standard 160 ml. separator bowl, rotating 40,000 R.P.M. (39,000*g* at the bowl periphery), at a rate of 14 liters per hour. The lysate was followed by 1 liter of chilled, sterile 0.9 per cent NaCl solution (adjusted to pH 6.5 with 0.1 *N* NaOH). The effluent fluid (clarified lysate) containing the phage, collected in a 10 liter Pyrex bottle, was ready for the concentration run in the Sharples centrifuge.

In later experiments it was found that filtration of the broth lysates through 10 inch 6 or 7 pound Mandler candles removed bacterial debris and flocculated material more efficiently, with less loss of phage activity, than did centrifugal clarification. This filtration technique was then used routinely in the preparation of broth lysate (filtered lysate) for subsequent centrifugation.

For the initial concentration of the phage, the lysate, clarified or filtered, was passed through the 50 ml. concentration bowl (20) of the Sharples centrifuge at a rate of 2 liters per hour. The bowl speed was 45,000 R.P.M. (49,000*g*). The lysate was followed by 1 liter of sterile 0.9 per cent saline, pH 6.5, at the same rate of flow. The bowl, plugged and capped (20), was shaken vigorously and generally set aside overnight in the refrigerator. The chilled bowl was again shaken thoroughly for 10 minutes, the 50 ml. of concentrate were poured into a sterile beaker, and the bowl was washed with three 20 ml. portions of sterile saline. The wash fluids were added to the concentrate.

The suspension was opalescent and showed a slight yellowish color due to a residual small amount of mucoid material, which, in contrast with that in fresh lysates, was in the form of finely divided, strand-like particles. This material was readily removed by angle centrifugation at 2000*g* for 10 minutes in 50 ml. lusteroid tubes. The resulting supernatant was siphoned off, and the pellets were washed into a single 15 ml. lusteroid tube with 10 ml. of saline and again angle centrifuged. The combined wash fluid and initial supernatant (Sharples angled concentrate), 120 to 250 ml., depending upon the volume of the starting material, showed a bright blue, opalescent Tyndall effect and were now devoid of yellow color. The angle centrifuge residue (Sharples angle residue) was suspended in 10 ml. of saline solution for subsequent titration and analysis.

The Sharples angled concentrate was distributed in 15 ml. portions and spun in the chilled rotor of the ultracentrifuge at 20,000*g* for 40 minutes. The supernatant (ultracentrifuge supernatant) was poured from the 3 to 4 mm. pellets of bluish, translucent, gel-like material. No extraneous material other than a few dust particles was seen, and the pellet of each tube was quickly dispersed in 1 or 2 ml. of saline solution by a few swirling motions. This suspension, in appropriate volume, was spun 10 minutes in the angle centrifuge at 2000*g*. The supernatant was pipetted off and the angle residue resuspended in 2 to 3 ml. of saline and angle centrifuged again. The wash fluid was combined with the concentrate (ultracentrifuge angled concentrate), while the angle residue was suspended in 10 ml. of saline for titration. The ultracentrifuge concentrate was strongly opalescent and blue, with sharp high lights when viewed by transmitted light.

The results of the biological titrations and of estimations of total nitrogen distribution in the various fractions, in a typical experiment with bacteriophage from a broth culture, are shown in Table I. The crude, unfiltered lysate contained $10^{9.87}$ plaque-forming particles per ml. Filtration through the Mandler candle in this case resulted in a relatively small loss of the phage activity, judging not only by the titer of the filtrate but by the titer of the Sharples angled concentrate and that of the ultracentrifuge concentrate. The titers of the Sharples angled concentrate and of the subsequent ultracentrifuge concentrate indicate an essentially complete recovery of the activity present in the filtered lysate. The losses shown by plaque counts of the Sharples effluent fluid, Sharples angle residue, ultracentrifuge supernatant, and angle residue are relatively negligible. It should be emphasized that, once a clarified *broth culture* lysate suitable for centrifugal purification is obtained, the phage activity can be sedimented in the ultracentrifuge repeatedly (at least three times) without appreciable loss. This is in marked contrast to the results with phage isolated from cultures in synthetic medium.

Isolation of Bacteriophage from Lysed Synthetic Medium Cultures

In cultures in synthetic medium, the plaque count reached about $10^{9.5}$ in 24 hours incubation and was maintained over the week of standing under refrigeration. The growth of phage-resistant bacteria during the longer incubation period resulted in a culture more readily clarified by centrifugation than by filtration. Consequently, synthetic medium lysates were usually clarified with the Sharples centrifuge, as described for the early experiments with broth lysates, though some (Table I) were filtered. The

TABLE I

Distribution of Nitrogen and Lytic Units in Purification Fractions of Broth and Synthetic Medium Cultures of Escherichia coli Infected with T₂ Bacteriophage

Fraction	Broth medium				Synthetic medium			
	Total volume	Total N	Titer*	Per cent activity	Total volume	Total N	Titer*	Per cent activity
	ml.	mg.			ml.	mg.		
Crude lysate.....	9000	9900	$10^{9.87}$	100	14,700	3350	$10^{10.80}$	100
Filtered lysate.....	9000	9180	$10^{9.74}$	70				
Clarified "					14,700	2910	$10^{10.43}$	84
Sharples effluent.....	9000	9140	$10^{8.38}$	3.2	14,700	2880	$10^{9.81}$	12.7
" angled concentrate.....	110	17.3	$10^{9.81}$	87.8	225	32.4	$10^{10.36}$	71.8
Sharples angle residue	10	0.7	$10^{6.7}$	0.07	12	9.0	$10^{3.55}$	2.1
Ultracentrifuge supernatant.....	110	2.7	$10^{7.97}$	1.3	225	2.5	$10^{8.08}$	0.4
Ultracentrifuge angled concentrate...	24.4	13.0	$10^{9.79}$	83.8	79.1	29.1	$10^{10.34}$	68.7
Angle residue.....	10.6	0.33	$10^{7.75}$	0.8	10.1	0.11	$10^{7.10}$	0.006

* All of the fractions were diluted on the basis of the original volumes of the crude lysate. Titer = number of plaque-forming phage particles per ml.

remainder of the purification procedure was the same as that used for the phage of broth cultures.

In Table I are shown the results of a typical experiment. The clarified lysate contained 84 per cent of the activity of the crude lysate. About 72 per cent of the activity was recovered in the Sharples angled concentrate; only about 3 per cent was lost during the following ultracentrifugal procedure.

Qualitative Chemical Examination

The final ultracentrifuge concentrates, from both broth and synthetic medium cultures, containing 2.0 to 8.0 mg. of phage per ml., showed a very marked Tyndall effect. The more dilute preparations were bluish; in

preparations of higher concentration, the bluish opalescence tended toward milkiness, and an orange tint was observed with transmitted light. Suspensions of broth phage in 0.9 per cent NaCl solution remained unchanged for long periods (2 to 3 months) without visible change or alteration in sedimentation characters and appearance in electron micrographs and with little, if any, diminution in infectivity. In comparable suspensions of synthetic medium phage, on the other hand, there soon appeared, in 1 to 2 weeks, a yellowish sediment and a marked increase in the viscosity of the fluid in association with changes in sedimentation characters, as noted below.

Concentrates containing 2 to 3 mg. of phage per ml., heated in the water bath at gradually increasing temperature (2° per minute), became clearer and more viscous at 58–60°. At 80–82°, the viscous preparation again became opalescent. This turbidity steadily increased, and distinct precipitation occurred at 90–92°. The precipitate tended to flocculate and, when cooled, the suspension was very viscous. When an equal volume of 0.1 N NaOH was added, the cooled suspension did not clear immediately, but did so after standing overnight at room temperature, about 25°. The phage precipitated immediately at H ion concentrations lower than pH 4.2 to 4.3. If the pH was not lowered below 3.0, the material redispersed above pH 4.5 without alteration of sedimentation characteristics. At about pH 10 and above, the opalescence disappeared, and the viscosity increased markedly.

The usual protein tests, biuret, ninhydrin, Millon's, xanthoproteic, were positive. The Molisch test was initially negative but became positive within 15 to 20 minutes, indicating the freeing of bound carbohydrate by hydrolysis. The Sakaguchi test was negative, as was the Hopkins-Cole. An Ehrlich benzaldehyde test for tryptophane, however, was weakly but definitely positive. A strongly positive pentose test with Bial's reagent (orcinol-HCl-FeCl₃) was obtained. Both the Schiff and diphenylamine tests were strongly positive. In addition, a strong Burgundy red color was obtained on heating 10 minutes in the water bath at 100° with tryptophane and perchloric acid (24). Spectrophotometric examination of an isoamyl alcohol extract of this chromogenic material revealed a curve characteristic of desoxyribose (sodium thymonucleate and yeast nucleic acid were used as controls).

The bacteriophage was precipitated on dialysis against distilled water. Addition of salt resulted in redispersion without change in the sedimentation or infectious characters observed with the material before dialysis. The salt-free material frozen and dried *in vacuo* was inactivated and was soluble with difficulty in 0.9 per cent NaCl solution, giving a preparation which was quite viscous and tended to froth badly.

Preliminary elementary and component analyses, with the methods used

in previous work (21) were made on the bacteriophage isolated from broth cultures, dialyzed against distilled water, then lyophilized and further dehydrated over P_2O_5 . The content of nitrogen was 13.5 per cent and that of phosphorus 4.8 per cent. The carbohydrate content, expressed as glucose, was 13.6 per cent. Estimation of nucleic acid by the method of Schmidt and Thannhauser (25) disclosed the presence of both desoxypentose and ribopentose nucleic acids. The amount of desoxypentose nucleic

TABLE II
Yield and Infectivity of Bacteriophage in Representative Experiments

Medium	Batch	Treat- ment	Lysate			Ultracentrifugal concentrate		
			Total volume	Titer*	Total N	Total N	Yield of phage per liter lysate	Infectivity (N per plaque- forming unit)
			<i>liters</i>		<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>gm.</i>
Broth	18	C.†	9.0	$10^{9.85}$	9810	7.10	5.85	$10^{-14.03}$
	27	F.†	9.4	$10^{9.89}$	8930	7.23	5.69	$10^{-15.89}$
	30	"	9.0	$10^{9.59}$	9180	13.0	10.69	$10^{-15.64}$
	31	"	9.0	$10^{9.86}$	9693	9.53	7.85	$10^{-15.89}$
	33	"	9.0	$10^{9.89}$	9702	11.6	9.54	$10^{-15.85}$
	34	"	9.0	$10^{9.73}$	8723	14.25	11.68	$10^{-15.70}$
Synthetic	22-3	C.	15.0	$10^{9.34}$	3213	2.22	1.05	$10^{-15.90}$
	24-5	F.	15.0	$10^{9.53}$	3120	3.62	1.79	$10^{-15.46}$
	29	"	7.22	$10^{10.47}$	1492	8.05	8.26	$10^{-16.00}$
	29A	C.	10.8	$10^{10.11}$	2332	14.5	9.89	$10^{-15.93}$
	32	"	14.7	$10^{10.43}$	2910	29.1	14.26	$10^{-15.70}$
Average†.....								$10^{-15.88}$

* Titer = number of plaque-forming phage particles per ml.

† F. = filtered through a 7 pound Mandler candle (see the text); C. = clarified in the Sharples centrifuge.

‡ Logarithm of the arithmetic average of the numbers corresponding to the individual logarithm.

acid was 40.0 per cent and that of ribopentose nucleic acid 6.6 per cent. Alcohol-ether-soluble material was also present to the extent of 5 to 6 per cent, of which 2.5 per cent was petroleum ether-soluble neutral fat.

The yield and infectivity of the purified phage from both broth and synthetic media are shown for several experiments in Table II. In all of these experiments the phage was sedimented twice, once in the Sharples centrifuge and then once in the vacuum ultracentrifuge. (As noted above, broth medium phage can be sedimented repeatedly, three times, with but small successive losses. In contrast, as observed in experiments not cited in Table II, repeated sedimentation of the synthetic medium phage results

in losses of 30 to 50 per cent of the activity with each succeeding sedimentation.) The yields showed little quantitative relation to the infectious titers of the lysates, except possibly in the instance of those from synthetic medium. The higher yields of 10 to 15 mg. per liter, obtained after experience with the purification process, are probably representative. There appeared to be no difference referable to preliminary filtration or clarification with the Sharples centrifuge for removal of bacteria from synthetic medium lysates.

The individual infectivity measurements of Table II were conducted at widely separated times, usually immediately after purification of the respective preparations. Nevertheless, the results appeared fairly uniform. The average amount of nitrogen per lytic unit was $10^{-15.53}$ gm., the average unit for broth phage being identical with that of phage from synthetic medium.

Sedimentation Characters

The bacteriophage exhibits a remarkable behavior hitherto not observed in ultracentrifugal studies of viruses. The material apparently may exist in two states with widely differing rates of sedimentation. Complete reversal in either direction can be accomplished by changes in the conditions of the dispersion media, or mixtures of the two states can be obtained at will. The T_2 phage of *Escherichia coli* shows an extraordinary power to absorb ultraviolet light, as Wyckoff (6) noted in the instance of the staphylococcus phage; excellent sedimentation diagrams were obtained with the T_2 bacteriophage at concentrations of 0.04 mg. per ml. Boundaries satisfactory for measurement of sedimentation rate resulted with concentrations of 0.013 mg. per ml.

The sedimentation studies reported here were all made in the pH region of about 6 to 7. The diagram of a concentrate of broth phage obtained in 0.9 per cent NaCl solution, as described above, is shown in Text-fig. 1. There is seen an exceedingly sharp sedimenting boundary above which there is no evidence of unsedimentable material, characters indicating a high degree of purity of the preparation by this method of examination. The sedimentation constant of this preparation was $S_{20} = 725 \times 10^{-13}$. Materials of these characters were obtained with only one exception in twenty experiments under the conditions described.

When, however, Ringer's solution was used as the dispersion medium for the purification process, the material, examined immediately, gave two boundaries, as shown in Text-fig. 2. The sedimentation constants in this instance were $S_{20} = 720 \times 10^{-13}$ and $S_{20} = 1031 \times 10^{-13}$. If such preparations were allowed to stand in the cold room for several days, the fast component became more prominent, while the more slowly sedimenting

component diminished in concentration. In some instances, the slow component disappeared completely. This change occurred without the appearance of unsedimentable material or alteration in the infectious capacity of the preparation.

Further study showed that the difference between the behavior in NaCl solution and that in Ringer's solution was probably due to the presence of calcium in the latter. It was found that the addition of CaCl_2 to a concentration of 0.023 M to a preparation of $S_{20^\circ} = 720 \times 10^{-13}$, similar to that of Text-fig. 1, effected an immediate complete transition to the state in which only the fast component was present. This is illustrated in Text-fig. 3, in which there is seen only the boundary $S_{20^\circ} = 1048 \times 10^{-13}$. The boundary of the fast component was usually not quite as sharp as that of the more slowly sedimenting component. The reverse transition of the fast



FIG. 1



FIG. 2

TEXT-FIG. 1. Sedimentation diagram of the bacteriophage suspended in 0.9 per cent saline solution. The concentration of phage was 1.1 mg. per ml. The mean centrifugal acceleration was $7230g$ and the time interval between pictures $2\frac{1}{2}$ minutes. The sedimentation constant was $S_{20^\circ} = 725 \times 10^{-13}$.

TEXT-FIG. 2. Sedimentation diagram of the bacteriophage suspended in Ringer's solution. The concentration of phage was 1.4 mg. per ml. The mean centrifugal acceleration was $7230g$ and the time interval between pictures $2\frac{1}{2}$ minutes. The sedimentation constants corresponding to the upper and lower boundaries were $S_{20^\circ} = 720 \times 10^{-13}$ and $S_{20^\circ} = 1031 \times 10^{-13}$, respectively.

component, present in 0.023 M CaCl_2 solution, to the slow component could be effected immediately by 10-fold dilution of the preparation with 0.9 per cent NaCl solution, thereby reducing the CaCl_2 concentration to 0.0023 M. That this was not related simply to dilution of the phage was evident, for the fast boundary was obtained routinely in 0.023 M CaCl_2 solution with a phage concentration as low as 0.04 mg. per ml.

In media of very low salt content, the phage did not give sharp boundaries of either sedimentation rate; instead, a broad band of diffuseness was seen in the region between the approximate limits expected of boundaries of $S_{20^\circ} = \text{about } 1000 \times 10^{-13}$ to $S_{20^\circ} = \text{about } 700 \times 10^{-13}$. Sharp boundaries were obtained without evidence of degradation of the material in high salt concentration, up to 18 per cent NaCl, or 15 per cent CaCl_2 .

As noted above, preparations of the phage from synthetic medium dispersed in NaCl solution undergo visible change in 1 to 2 weeks in the refriger-

erator. Sedimentation diagrams showed a diminution in the material of $S_{20}^\circ = \text{about } 700 \times 10^{-13}$ in association with the appearance of a new sharp boundary of $S_{20}^\circ = 14 \times 10^{-13}$. Further changes toward degradation resulted in the appearance of unsedimentable material.

The finding of two boundaries associated with the fully infectious material raised the question of the state in which the phage exists in the lysates. This was investigated first by direct examination of lysates of *Escherichia coli* grown in synthetic medium, an examination possible because of the high concentration of the phage, its high ultraviolet-absorbing power, and the low ultraviolet absorption of the medium itself. In Text-fig. 4 there is shown a distinct sharp boundary with a sedimentation constant of $S_{20}^\circ = \text{about } 1000 \times 10^{-13}$. As expected, there was present, also, much unsedimentable material (probably due to bacterial degradation products).



FIG. 3



FIG. 4

TEXT-FIG. 3. Sedimentation diagram of the bacteriophage obtained in the presence of 0.023 M CaCl_2 in the suspending medium. The concentration of phage was 1.4 mg. per ml. The mean centrifugal acceleration was $7230g$ and the time interval between pictures $2\frac{1}{2}$ minutes. The sedimentation constant was $S_{20}^\circ = 1048 \times 10^{-13}$.

TEXT-FIG. 4. Sedimentation diagram of bacteriophage in a crude synthetic medium lysate. The titer of the lysate was $10^{12.6}$ plaque-forming units per ml. The mean centrifugal acceleration was $7230g$ and the time interval between pictures $2\frac{1}{2}$ minutes. The sedimentation constant was $S_{20}^\circ = \text{about } 1000 \times 10^{-13}$.

Further evidence that the state resulting in the boundary $S_{20}^\circ = \text{about } 1000 \times 10^{-13}$ is representative of the phage in the lysates was shown by comparing the sedimentation of the phage infectivity of a broth lysate with that of the influenza virus B, which has a sedimentation constant of $S_{20}^\circ = 824 \times 10^{-13}$. The procedures employed were those described (26, 27) in a similar comparison of influenza viruses A and B with the papilloma virus. The results showed that the phage infectivity sedimented at a rate much greater than that of influenza virus B, a rate more compatible with phage particles of $S_{20}^\circ = \text{about } 1000 \times 10^{-13}$ than of $S_{20}^\circ = \text{about } 700 \times 10^{-13}$.

Electron Micrography

Like most other viruses, the bacteriophage in purified preparations was very difficult to portray in the electron microscope. The chief difficulty, that of obtaining an even distribution of a large number of particles on the collodion screen, was surmounted by the addition of calcium chloride to the

suspending medium as in the work with the equine encephalomyelitis (28) and influenza viruses (26, 27). By this means, the contrast in the images was likewise greatly increased, though the internal structure was entirely obscured.

Electron micrographs of the purified T₂ bacteriophage from broth culture lysates, obtained both in the conventional way and by the "shadow" technique (29), have been shown in a previous report (15). This phage, as observed (10) in crude lysates, is a tadpole-shaped particle with a large head, essentially a short rod (15) with conical or rounded ends, and a slender tail terminating distally in a slightly broader knob or disk-shaped structure. A micrograph of particles obtained from a broth lysate and treated with calcium chloride in 0.023 M concentration is portrayed in Fig. 1. In Fig. 2 there are shown the particles obtained from a lysate in synthetic medium. On inspection, it is seen that the particles from the two types of lysates are closely similar; a significant difference, however, is clearly evident in the length, especially of the tail structure. That of the phage from synthetic medium lysates was significantly longer than that of the virus from broth cultures. Measurements reveal that the head pieces are likewise somewhat larger. The values of the sizes from measurements on small numbers of the images from broth lysates are length of head piece 100 m μ , and width 80 m μ ; length of tail piece 111 m μ , and width 18 m μ ; and over-all length 211 m μ . Analogous values for phage from synthetic media were 113 and 86 m μ ; 135 and 20 m μ ; and 248 m μ . It should be emphasized that these values were obtained with the particles dried in the air and then in the vacuum of the electron microscope, and may not represent the true values for the particles in aqueous medium.

In order to observe the internal structure in the particles of purified preparations, like that seen in the particles of crude lysates (10), efforts were made to obtain micrographs in the absence of calcium. As shown in Fig. 3, fair success resulted with particles from which most of the salt had been dialyzed with distilled water. The contrast in the images is low, and the limits of the particles are indistinct. Inside the particle, however, there are seen regions, usually two, of segregated material of relatively high electron absorption. Agglomeration, observed macroscopically in dialyzed concentrates, is likewise seen here. Washing with distilled water of particles dried on the screen from salt solutions sometimes yielded the bizarre forms seen in Fig. 4. The particles appear greatly swollen and rounded, the periphery is fairly distinct, and the characteristic internal structure seems faded. Such an appearance might result from rapid imbibition of water by and swelling of the previously dried particles. This possible osmotic effect could occur if it were assumed that a large amount of salt was taken up by the particles during the course of the drying process. Such

a possibility is not inconceivable, for, as indicated below, the particles are apparently enclosed within a membrane-like structure. To what extent the images represented artifacts cannot be judged at present.

A micrograph of particles dried from CaCl_2 solution and shadowed with chromium is given at low magnification in Fig. 5, chiefly to show the preponderance of characteristic forms and the absence of significant numbers of small particles of other sorts. A somewhat different appearance of the shadowed bacteriophage was obtained with the virus, the same preparation as that of Fig. 3, from which the salts had been removed by dialysis against distilled water. In this instance the particles were shadowed with gold. The result is shown in Fig. 6, where it is seen that the surface contours of the particles are not smooth; instead, there is unevenness, which gives the appearance of protrusion of internal structures beneath the surface. The shape and location of the apparent surface mounds correspond closely to those of the regions of high electron-absorbing power seen in the conventional electron micrograph of Fig. 3. Analogous surface protrusions have been seen in micrographs of shadowed elementary bodies of vaccinia (15).

A most interesting and probably significant finding is illustrated in the micrograph of Fig. 7. Here there are seen many images of characteristic outline but seemingly empty with respect to the electron-absorbing material of the head piece, as previously observed by Anderson (14). These forms or ghosts have the appearance of limiting, membrane-like structures from which the internal contents have escaped or have undergone profound changes. Similar forms, less clearly seen, are present also in Figs. 1 and 2. Structures somewhat analogous to these are the ghost forms seen in preparations of vaccinia elementary bodies (30), some of which have apparently ruptured, spilling the contents to the outside. It is notable that the calcium does not greatly enhance the contrast of these limiting structures of the bacteriophage, which suggests that its action may be exerted on the material contained inside the particle.

DISCUSSION

The application of centrifugal purification procedures to cultures of *Escherichia coli* grown in broth or synthetic medium and lysed by the T₂ bacteriophage has been consistently productive of a material which exhibits uniformly the biological behavior of the bacterial virus. The yields of activity from both broth and synthetic medium lysates were high, representing in some instances about 85 per cent of that initially present in the crude lysates. The rate of sedimentation of the activity from the lysates by the Sharples supercentrifuge and the ultracentrifuge corresponded well with that of the purified material examined in the analytical ultracentrifuge.

Though infectivity measurements made on the purified material cannot be employed as a quantitative index of purity, the values of the infectious units of the various preparations were highly uniform.

The sharpness of the two separate boundaries observed in the analytical ultracentrifuge, together with the absence of unsedimentable material in the sedimentation diagrams, constitutes evidence of a high degree of homogeneity of the material with respect to particle size, shape, and density. The small particles of 2 to 8 $m\mu$ in diameter, indicated by diffusion methods (9), were not present in sufficient amounts for detection in the sedimentation diagrams. Further, the centrifugal fields employed for purification were not great enough for the differential selection of particles of these dimensions. In the electron micrographs, only one type of particle was consistently present in more than a trace. While particles of 2 to 8 $m\mu$ in diameter might be visualized only with difficulty, if at all, in the electron microscope, particles of 12 $m\mu$ or more in diameter would have been demonstrable, if present in significant amount, especially in the shadow micrographs.

Small particulate components from the tissues or fluids of the host have provided complications in the purification of other viruses (31, 32), but in each instance the methods of ultracentrifugal analysis or electron micrography have sufficed to demonstrate (26, 28) their presence. The doubtful reliability of diffusion methods for the estimation of virus particle size was clearly shown in the instance of the influenza virus A (PR8 strain), which was judged by this method to be about 9 $m\mu$ in diameter (33) and which was subsequently found (34, 35) by other means of measurement to be about 100 $m\mu$ in diameter (an average value). Furthermore, the intimate biological relationship between the virus particle of characteristic shape and size and its bacterial host has been beautifully shown in repeated studies by Anderson and his associates (10, 14). A consideration of the various ultracentrifugal and electron micrographic data together with the biological findings provides a preponderance of evidence that the characteristic particles of the purified preparations are identical with the T_2 bacteriophage.

A purpose of the present work was to obtain data of possible significance in interpretations of the nature of bacteriophage, which has been regarded by many as a macromolecular protein (9) or nucleoprotein (5). Such interpretations imply that phage is an inanimate entity devoid of autonomy and dependent on the host cell for the immediate vital processes necessary for reproduction of the phage particles. Interpretations in this vein have been implemented by frequent comparisons (5, 9) of the characters of the bacteriophage with those of enzymes and autocatalytic enzymes. It is notable, however, that no proof of molecular nature has been reported; instead, there are available only negative findings pertaining to failure to demonstrate vital processes usually considered as criteria of living matter.

Certain of the data obtained with the T_2 bacteriophage provide presumptive evidence against the molecular hypothesis. Chemical analyses, described briefly here and in detail in another report (36), show that the agent consists of protein, lipid, and nucleic acid of two types. The electron microscope reveals that the constituents are arranged in the particle in a highly organized and differentiated manner. The material within the head piece shows not only segregated regions of high electron-absorbing power surrounded by material of low absorbing power, but differentiation as well from a limiting structure which is left behind like a membrane still attached to the tail process when the material escapes to the outside. The chemical constitution of the whole virus particle and the appearances of organization are no less complex than the analogous characters of the host organism (36). In view of the exhibition of clearly defined differentiation, it seems improper to refer to the bacteriophage as a nucleoprotein; rather, like its host and other living cells, it is an organized complex of these constituents. In addition, the complex is subject to change in size, as shown in the electron micrographs, and in chemical constitution in response to the nature of the medium in which the host grows. The quantitative changes in constitution do not parallel analogous variations in the host (36) in response to different environments. Despite these significant changes, however, the virus maintains the fundamental characterizing capacity to parasitize and increase in the presence of the specific host.

An explanation of the two distinct boundaries of greatly different rate of sedimentation, both associated with the same material of constant activity, is not apparent with the present information. The phenomenon has been observed in relation not only to the presence of calcium but also to hydrogen ion concentration, as described in another report. There is some reason (37) to believe that the boundary characterized by $S_{2.0} =$ about 1000×10^{-13} represents the phage dispersed as individual particles, or possibly as aggregates of one type of cohesion, while that of $S_{2.0} =$ about 700×10^{-13} is in a state of aggregation of another type consisting of two or more particles per group. The reason for the effect of calcium in converting the material of $S_{2.0} =$ about 700×10^{-13} into that of $S_{2.0} =$ about 1000×10^{-13} is obscure. No evidence has been seen of the splitting of the particles of $S_{2.0} =$ about 1000×10^{-13} into smaller particles, nor have electron micrographs been obtained of aggregates of pairs or other multiples of particles occurring uniformly. The phenomenon was not observed by Wyckoff (6) with the phage of staphylococcus, nor were the gel preparations occurring in his work encountered with active preparations of the T_2 phage. The problem of the two boundaries of the T_2 phage will be discussed in further detail in association with a report (37) of the studies of the effects of hydrogen ion concentration.

SUMMARY

The T₂ bacteriophage of *Escherichia coli* (strain B) has been purified by preliminary filtration or spinning of lysates in broth or synthetic medium in the Sharples centrifuge for removal of bacterial debris, followed by sedimentation of the virus in the Sharples centrifuge and further concentration of the agent in the vacuum ultracentrifuge. The concentrates possessed uniformly the specific biological activity of the T₂ bacteriophage in its action on the host. In the analytical ultracentrifuge, the fully active concentrates in 0.9 per cent NaCl solution as the suspending medium gave a single sharp boundary, one with a sedimentation constant of $S_{20}^0 =$ about 700×10^{-13} ; in 0.023 M CaCl₂ solution, a single, slightly less sharp boundary was obtained with a sedimentation rate corresponding to $S_{20}^0 =$ about 1000×10^{-13} . The two states were fully reversible by suitable changes in the medium, or mixtures of the two states could be obtained as desired. No difference was seen between the sedimentation rates of the virus from broth and that from synthetic medium. The purified bacteriophage obtained from broth lysates appeared more stable than that from synthetic medium; the latter began to disintegrate within 1 to 2 weeks, the suspension became viscous, and a new component of $S_{20}^0 =$ about 14×10^{-13} appeared. Further degradation was associated with the appearance of unsedimentable material. In the electron microscope, there were seen characteristic tadpole-shaped particles of 211 m μ total length and 80 m μ width of head piece for the broth bacteriophage, and 248 and 86 m μ for the bacteriophage from synthetic medium. There was definite morphological differentiation in internal structure. Some of the images were of the characteristic outline but devoid of the contents of the head piece, having the appearance of limiting, membrane-like structures.

Chemically, the agent from broth lysates consisted of protein 50.6 per cent, lipide 5 to 6 per cent, and carbohydrate 13.6 per cent. There was present 40 per cent nucleic acid, of which 33.4 per cent was of the desoxypentose and 6.6 per cent of the ribopentose type. The nitrogen and phosphorus contents of the whole complex were 13.5 and 4.8 per cent, respectively. The best yields of the virus were 10 to 15 mg. per liter of either broth or synthetic medium lysates. The unit of infectivity contained $10^{-15.88}$ gm. of nitrogen.

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EXPLANATION OF PLATES

PLATE 1

FIG. 1. Bacteriophage from a broth lysate of *Escherichia coli*. The purified phage suspension placed on the collodion film contained 0.023 M CaCl_2 . Magnification 11,000 \times .

FIG. 2. Bacteriophage from a synthetic medium lysate of *Escherichia coli*. The purified phage suspension placed on the collodion film contained 0.023 M CaCl_2 . Magnification 11,000 \times .

FIG. 3. Bacteriophage from a broth lysate of *Escherichia coli*. The suspension placed on the collodion film had been freed of salt by dialysis against distilled water at 2-8°. Magnification 11,000 \times .

PLATE 2

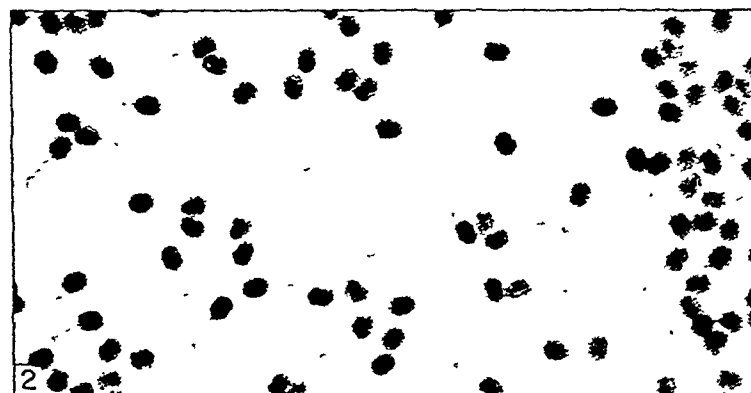
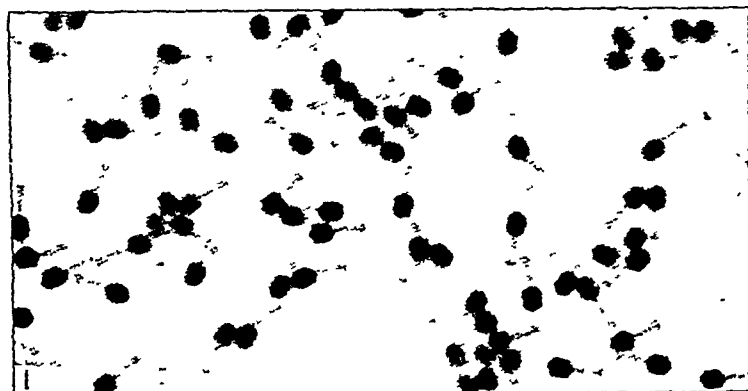
FIG. 4. Bacteriophage from a broth lysate of *Escherichia coli*. The bacteriophage suspension in 0.9 per cent NaCl solution was diluted with 15 volumes of distilled water before it was placed on the collodion film. After drying, the film was washed with distilled water. Magnification 11,000 \times .

FIG. 5. Bacteriophage from a broth lysate of *Escherichia coli*, prepared as for Fig. 1 and then shadowed with chromium at the angle whose tangent is $\frac{1}{2}$. Magnification 17,700 \times .

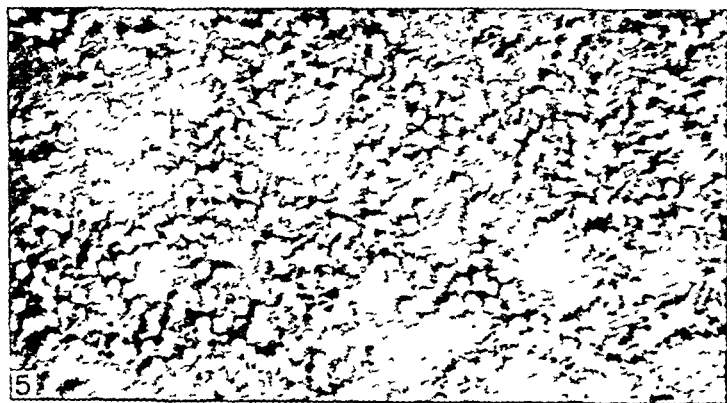
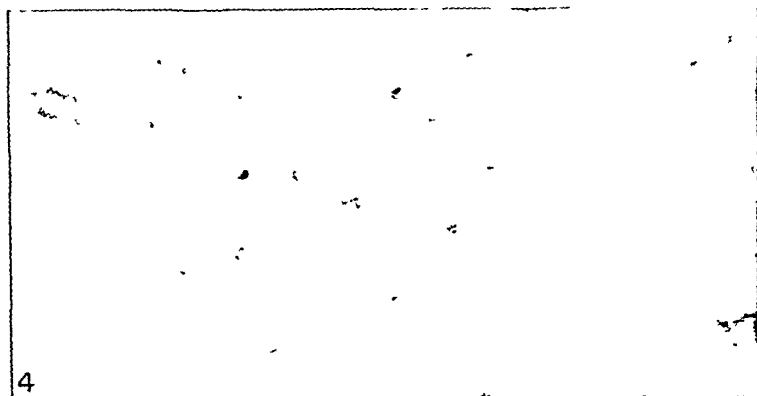
PLATE 3

FIG. 6. Bacteriophage from the same preparation as Fig. 3. The dialyzed bacteriophage, after drying on the collodion, was shadowed with gold at the angle whose tangent is $\frac{2}{3}$. Magnification 11,000 \times .

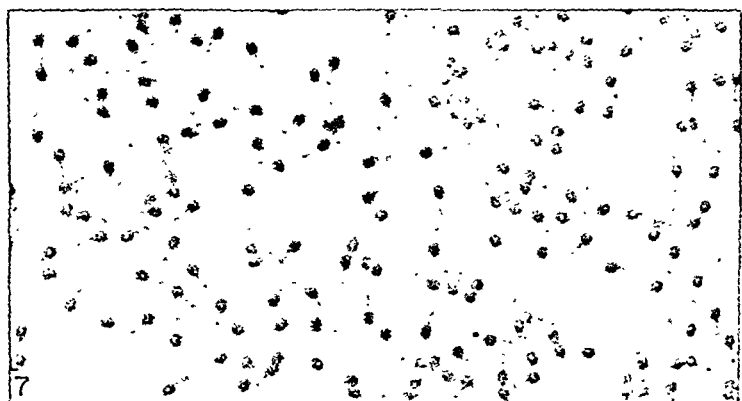
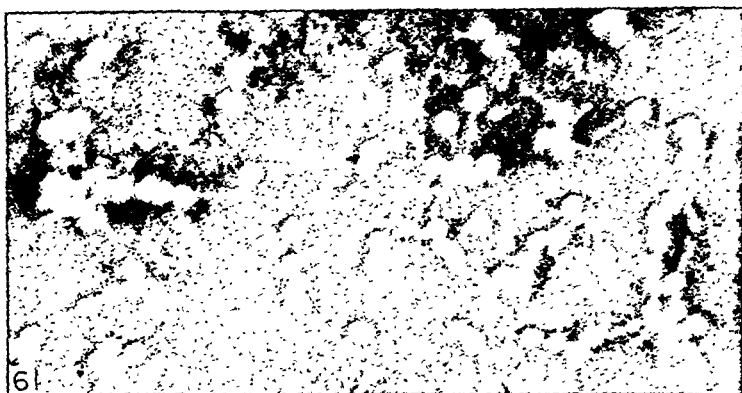
FIG. 7. The same preparation as that of Fig. 1 shown at the magnification 17,700 \times .



(Hook, Beard, Taylor, Sharp, and Beard: Isolation of bacteriophage)



(Hook, Beard, Taylor, Sharp, and Beard Isolation of bacteriophage)



(Hook, Beard, Taylor, Sharp, and Beard. Isolation of bacteriophage)

SEDIMENTATION CHARACTERS AND pH STABILITY OF THE T₂ BACTERIOPHAGE OF ESCHERICHIA COLI*

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In accompanying reports, there are described the purification and certain of the physical and chemical characters (1, 2) of the T₂ bacteriophage of *Escherichia coli*, strain B, obtained from lysates of the host organism grown in both broth and synthetic media.

In further work on the virus, studies were undertaken to determine the effects of hydrogen ion concentration on the stability of virus infectivity, in parallel with an examination of the physical stability of the virus particle under like conditions, as determined in sedimentation diagrams. In previous studies (1) there were observed two distinct and greatly different rates of sedimentation of the fully active virus under different conditions. When the bacteriophage was suspended in 0.9 per cent NaCl solution, a sedimentation rate of $S_{20}^0 = \text{about } 700 \times 10^{-13}$ was observed; in the presence of 0.023 M CaCl₂, $S_{20}^0 = \text{about } 1000 \times 10^{-13}$ was found. The two states could be reversed, or a mixture of the two could be obtained by appropriate changes in the suspending medium. In the work described in this paper, there was encountered an identical phenomenon of the same two sedimentation rates, but unrelated, in this instance, to the presence of calcium. In media more acid than pH 5.8, the more rapidly sedimenting component alone was observed, while above this pH region only the slower one was seen. The present report is concerned with the results of these studies on the effects of hydrogen ion concentration on the infectivity and physical behavior of the virus particle. There are described, also, experiments to study the effects of virus concentration on the rate of sedimentation.

Materials and Methods

The T₂ bacteriophage (3), which has been designated as P. C. (4) and γ (5), was purified as previously described (1). The agent, obtained from lysates of *Escherichia coli* grown in nutrient broth, was employed

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for the studies on pH stability, since, as already noted (1), it appears more stable, after purification, than that derived from lysates in synthetic medium.

The buffer solutions for the studies on the stability of infectivity were prepared by dissolving 2.051 gm. of sodium acetate (0.025 M), 5.152 gm. of sodium veronal (0.025 M), and 2.922 gm. of sodium chloride (0.05 M) in nearly 1 liter of distilled water. In making the individual solutions to 1 liter, HCl or NaOH was included to give the desired pH. A series of buffers, at successive intervals of approximately 1 pH increment, was prepared in the range between pH 2 and 11.

The buffers of various pH values were distributed in 18 ml. volumes in 25×250 mm. Pyrex test-tubes, and, to each, 2.0 ml. of a concentrate containing 5.5 mg. of phage per ml. were added. The tubes were tightly stoppered and kept immersed to about half their height, well above the level of the liquid in them, in a melting ice-water bath at about 0.5° during the course of the experiment. The virus mixtures were not sterile, but precautions were observed to avoid excessive bacterial contamination. There was set up, also, a control preparation in which the virus was suspended in a solution of 0.9 per cent NaCl and 0.1 M phosphate at pH 7.0.

Immediately before the preparation of the mixtures, the stock phage concentrate was titrated (1) and the result used as a basis for calculating the extent of the subsequent decline in activity of the test preparations. After various time intervals, a sample of 0.5 ml. was removed from each mixture and from the control preparation, and the infectivity was determined. Another sample of like volume was removed, also, for pH determination with the glass electrode. The mixtures were not corrected to their original pH during the experiment.

The purified broth bacteriophage used for most of the studies concerned with the effect of pH on the sedimentation characters of the virus was a part of a large amount prepared for chemical analysis (2). The results of experiments made with other phage preparations, also, have been used to support some of the generalizations from the experiments described in detail here. In all, 62 sedimentation diagrams were obtained.

Sedimentation rates were measured by means of an air-driven ultracentrifuge (6) whose rotor carried a sector-shaped cell of 5° angle and 12 mm. height at a mean radius of 6.5 cm. All runs, except one described below, were made at 167 R.P.S., giving a centrifugal field in the cell at mean radius of 7230*g*. Boundary progress was recorded by means of the ultraviolet light absorption method of Svedberg. Liquid thickness in the light beam (cell thickness) was 5 mm. for all runs. The time interval between exposures, except in one instance, was $2\frac{1}{2}$ minutes.

The sedimentation rate of the bacteriophage was examined at pH 3.6

to 5.8 in acetate buffer, at pH 5.8 to 7.6 in phosphate buffer, and at pH 8.6 to 10.4 in borate buffer. The buffers were made to 0.2 M concentration in a salt solution containing 9.0 gm. of NaCl and 1.0 gm. of KCl per liter. All solutions were kept in the refrigerator at 2-8° until a run was to be made. The stock virus preparation was diluted to contain 1.25 mg. of bacteriophage per ml. with the NaCl-KCl salt solution; further dilution in equal parts with the appropriate 0.2 M buffer-saline solution brought the sample to 0.625 mg. of virus per ml. and 0.1 M concentration of buffer at the desired pH, the same concentration of NaCl and KCl having been maintained throughout. This sample was then raised to room temperature (about 23°) before the centrifuge cell was filled. The time elapsing between preparation of each mixture and analysis, in all instances, was about 15 minutes.

The studies of the effect of virus concentration on sedimentation rate were made on bacteriophage also derived from a broth lysate. The range of bacteriophage concentration was 0.013 to 3.7 mg. per ml. Because of the different sedimentation rates related to hydrogen ion concentration, a study was made at pH 4.6 ($S_{20}^0 = \text{about } 1000 \times 10^{-13}$) and another at pH 6.6 ($S_{20}^0 = \text{about } 700 \times 10^{-13}$). The suspending media employed at the respective pH regions were the same buffer-salt solutions used at the corresponding pH in the stability studies described above. Sedimentation rates have been reduced, wherever possible, to the standard conditions of the density and viscosity¹ of water at 20°.

Results

The effects of hydrogen ion concentration on stability of infectivity of the bacteriophage are shown in Fig. 1. There occurred a decline in activity throughout the whole pH range studied, but a broad pH region of stability was seen between 5 and 9, with an apparent maximum at about pH 5 to 6. Somewhat less stability was apparent in the region near pH 7.0, but this may have been related to adverse action of the acetate or veronal buffer salts at this hydrogen ion concentration. The unattached points of Fig. 1, which indicate the activity of the control preparation, show a decline in infectivity at this pH no greater than that occurring in the region of maximum stability at about pH 5. It is notable that no discontinuity in either the general level of infectivity or stability was observed in the region of pH 5.8, where the level of sedimentation constants changed, as described below.

At pH 2.0, the phage coagulated immediately upon contact with the buffer; at pH 3.0, it precipitated immediately, but did not coagulate. At

¹ The viscosity measurements of the salt and buffer solutions were made through the generous cooperation of Dr. Hans Neurath.

the end of 4 hours, the phage at pH 4.0 was precipitated but not coagulated. Further tests showed that precipitation of the phage occurred at pH 4.25 but not at pH 4.50. The phage precipitating at pH 3.0 and 4.0 could be easily redispersed by shaking, and observation of the precipitate under the light microscope revealed amorphous aggregates. At pH 11.0, the suspension of phage immediately lost its characteristic blue opalescence.

The pH of the virus-buffer mixtures remained relatively constant at pH 8.0 and below, but decreased steadily above 8.0, so that at the end of the experiment the buffers at pH 9, 10, and 11 had changed to pH 8.6, 8.93, and 9.51, respectively.

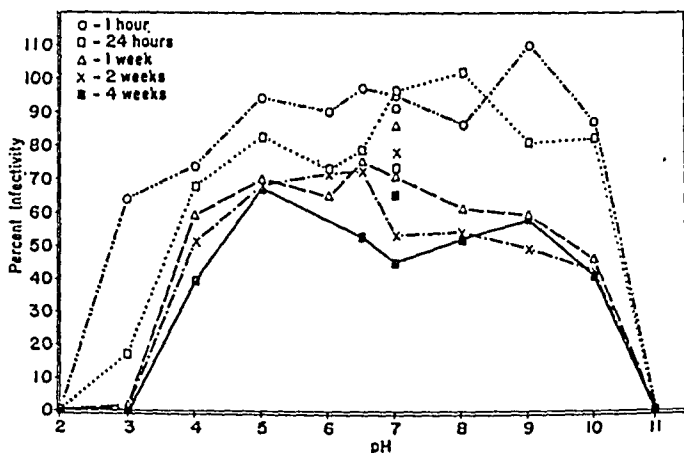


Fig. 1. pH stability of the bacteriophage of *Escherichia coli* isolated from broth lysates.

In Table I there are given the data obtained from the experiments concerning the effects of hydrogen ion concentration on the sedimentation characters of the virus. The sedimentation rates are clearly grouped at two levels, the one at $S_{20}^0 = \text{about } 1000 \times 10^{-13}$ existing in the region pH 4.6 to 5.8, and the other, at $S_{20}^0 = \text{about } 700 \times 10^{-13}$, extending from pH 5.8 to 9.5. The sedimentation diagram of a preparation at pH 4.6 is given in Fig. 2. In this instance $S_{20}^0 = 1044 \times 10^{-13}$, and the sedimentation characters of the preparation are like those indicated in the diagram previously obtained, Text-fig. 3 of (1), in the presence of 0.023 M CaCl_2 at a pH of about 6.5. The diagram in the buffer-salt solution at pH 6.6 shown in Fig. 3, $S_{20}^0 = 708 \times 10^{-13}$, does not differ from the diagram, Text-fig. 1 of (1), obtained with the virus suspended in 0.9 per cent NaCl solution at a pH of about 6.5. The transition point between the two levels of sedimentation constants was close to pH 5.8. At this pH, both of the boundaries were observed in the same sedimentation diagram.

TABLE I
Sedimentation Constants of T_2 Bacteriophage of *Escherichia coli* in Relation to Hydrogen Ion Concentration

pH	Buffer	$S_{20}^0 \times 10^{12}$	Remarks
3.6	Acetate		Insoluble
4.6	"	1044	Sharp, single boundary (Fig. 2)
5.6	"	1002	Slightly diffuse, single boundary
5.8	Phosphate	1016	Very faint
		750	Predominant, slightly diffuse
6.1	"	698	Sharp, single boundary
6.6	"	708	" " " (Fig. 3)
7.6	"	694	" " "
8.6	Borate	713	" " "
9.5	"	726	" " "
10.4	"	448*	Faint, very sharp boundary (Fig. 4)
		13.4	Predominant, very sharp boundary (Fig. 4)

* These particular values were not corrected for the relative viscosity or density of the suspending medium. See the text.



FIG. 2

FIG. 2. Sedimentation diagram of the bacteriophage at pH 4.6. The sedimentation constant was $S_{20}^0 = 1044 \times 10^{-12}$.



FIG. 3

FIG. 3. Sedimentation diagram of the bacteriophage at pH 6.6. The sedimentation constant was $S_{20}^0 = 708 \times 10^{-12}$.



FIG. 4

FIG. 4. Sedimentation diagram of the bacteriophage preparation at pH 10.4. Two boundaries are present; for that at (a) $S = 13.4 \times 10^{-12}$ and that at (b) $S = 448 \times 10^{-12}$. These values are uncorrected for viscosity and density of the suspending medium for reasons given in the text.



FIG. 5

FIG. 5. Sedimentation diagram of the slow component (a) of Fig. 4. The mean centrifugal acceleration in this instance was $29,840g$ and the time interval between pictures 5 minutes. The sedimentation constant was $S = 13.4 \times 10^{-12}$ (not corrected for viscosity and density of the suspending medium).

Some additional experiments were made to extend the investigation beyond the effective limits of the buffers used. pH was increased by adding NaOH to a borate-buffered sample until a change in the sedimentation characters was brought about at pH 10.4. The diagram of Fig. 4, obtained at this pH, shows two boundaries, $S = 448 \times 10^{-13}$ and $S = 13.4 \times 10^{-13}$. Most of the light-absorbing material was the more slowly sedimenting component, just beginning to show beneath the meniscus in Fig. 4. This slowly sedimenting material, which seemed viscous and which exhibited a strong capacity for the absorption of ultraviolet light, is clearly demonstrated in the sharp boundary seen in Fig. 5. Inasmuch as viscosity determinations on the small volume of the supernatant fluid of this run were not made, properly corrected sedimentation constants could not be calculated for the boundaries of either Fig. 4 or Fig. 5. Titrations of lytic capacity under these conditions showed considerable activity remaining, Fig. 1, and it is possible that the boundary $S = 448 \times 10^{-13}$ is the remainder of the material of $S_{20^\circ} = \text{about } 700 \times 10^{-13}$, sedimenting at a retarded rate through the apparently viscous breakdown products of the major part of the phage.

At pH 3.6 and less, where the phage precipitated immediately, there was no evidence of light-absorbing material in the supernatant examined with the ultracentrifuge. Although the phage precipitated at pH 3.6, its lytic activity was not destroyed for some time, as shown in Fig. 1. There was, likewise, no evidence of change in physical stability under these conditions, for when the precipitate was taken up in 0.1 M phosphate buffer-saline at pH 6.6, the boundary $S_{20^\circ} = \text{about } 700 \times 10^{-13}$ was obtained in the ultracentrifuge. This boundary was indistinguishable from that of Fig. 3. Another sample, adjusted to pH 1.7 with HCl, then returned to pH 7.5 with NaOH, did not show any boundary or characteristic blue opalescence.

It has already been seen (1) that the bacteriophage absorbs ultraviolet light strongly and can be photographed in sedimentation diagrams at concentrations as low as a few micrograms per ml. Furthermore, dilution by a factor of 5 below the concentration (0.625 mg. per ml.) used in these experiments causes only a slight change in sedimentation rate under otherwise similar conditions, as described below. A sample of bacteriophage at pH 4.6 in 0.1 M acetate buffer-saline solution, where sedimentation occurred at $S_{20^\circ} = 1044 \times 10^{-13}$, was diluted with 4 volumes of 0.2 M phosphate buffer-saline at pH 7.6. The resulting pH was 7.2, and the boundary changed to $S_{20^\circ} = 723 \times 10^{-13}$, with no evidence of damage to the virus.

Inasmuch as the boundary $S_{20^\circ} = \text{about } 1000 \times 10^{-13}$ was seen in the present work only in those samples which contained acetate buffer, it was considered possible that some effect, other than pH alone, brought about specifically by the acetate buffer, might be responsible for the difference in

sedimentation rates. Even though no such discontinuity occurred in passing from phosphate to borate buffer in the alkaline range, a different buffer was tried to bridge the gap from pH 4.6 to 6.6. For this purpose a citrate-phosphate buffer effective over this range was used. In this buffer, 0.1 M concentration in the NaCl-KCl saline solution as described above, the boundaries were as before; i.e., S_{20}^0 = about 1000×10^{-13} at pH 4.6 and S_{20}^0 = about 700×10^{-13} at pH 6.6.

In work previously reported (1) it was found that the addition of 0.023 M CaCl_2 to phage suspensions of S_{20}^0 = about 700×10^{-13} caused an immediate change to S_{20}^0 = about 1000×10^{-13} . These experiments were carried on in the range of pH 6.0 to 7.5. In the present work similar addition of CaCl_2 (0.023 M) to acetate-buffered preparations of phage at pH 4.6 had no effect on the sedimentation constant. It appears that no discontinuity in sedimentation rate from S_{20}^0 = about 1000×10^{-13} at or below 5.8 to S_{20}^0 = about 700×10^{-13} above this point would exist if 0.023 M CaCl_2 were present in all samples.

The effects of the concentration of bacteriophage on sedimentation rate are shown graphically in Fig. 6. In the upper curve, obtained at pH 4.6, there is seen a linear dependence of sedimentation rate on virus concentration in the range of 3.7 to 0.125 mg. of bacteriophage per ml. The next dilution, 0.041 mg. per ml., reveals a significant decrease in the sedimentation constant. Qualitatively similar results were obtained at pH 6.6, as shown in the lower curve of Fig. 6. Again the linear portion extends from 3.7 to 0.125 mg. of bacteriophage per ml.; at 0.013 mg. of phage per ml., the sedimentation constant was sharply decreased. It is observed that the slopes of the two linear relations were not identical.

A means was not at hand for study of the factors responsible for the two sedimentation rates of the bacteriophage directly on the particles themselves under the various conditions. Since there was no evidence of change in infectious capacity in association with change in sedimentation rate, it seemed possible that both boundaries represented intact particles in different states of dispersion or aggregation. This was investigated indirectly by the examination of the behavior of lucite models of the bacteriophage sedimenting individually and in various combinations of two or more units.

The models were made in the shape of the phage particles to scale measurements taken from electron micrographs (1). The over-all length of each, *A* of Fig. 7, was 2.5 cm., and the models, made on a lathe, possessed cylindrical symmetry. The models were allowed to sediment in a glass vessel containing NaCl solution, the density of which was about 1.18. Under these conditions the motion was slow enough to avoid large acceleration errors, and it was possible to observe the orientation of permanent

translation as well as the sedimentation rate. In Fig. 7 are seen the individual model and the various combinations made by sticking together the individual units with Duco cement. The respective lengths of time necessary for sedimentation through a vertical distance of 27 cm. are also given, as are reciprocals of these values adjusted by constant multiples to 100 for a single unit.

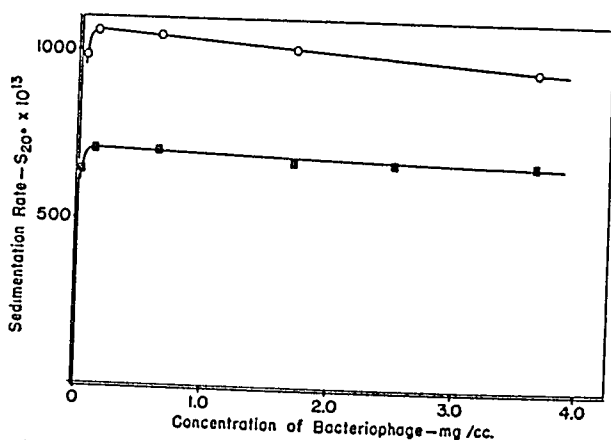


FIG. 6. Relation of sedimentation constants of the bacteriophage to bacteriophage concentration at pH 4.6 (open circles) and 6.6 (closed squares).

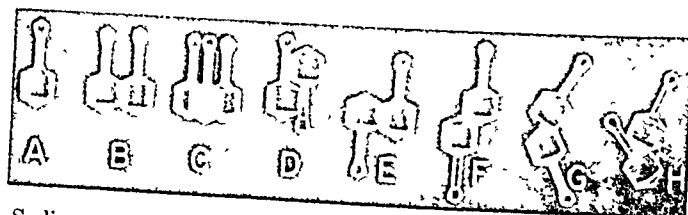


FIG. 7. Sedimentation characters of lucite models of the T_2 bacteriophage of *Escherichia coli* in concentrated NaCl solution, singly and in various simple combinations. The time of sedimentation, in seconds, was 7.9, 8.5, 8.4, 11.5, 11.3, 11.4, 10.9, and 11 (approximate, see the text) for A through H respectively; the corresponding sedimentation rate, in arbitrary units, was 100, 93, 94, 69, 70, 69, 72, and 72.

In the data of Fig. 7, two general groups of sedimentation rates are apparent. The greatest rate of sedimentation was that of the individual unit, A. Nearly the same (only 6 or 7 per cent less) were the values of arrangements B and C, where all tails were in the same direction. All gave values of 69 to 72, about 30 per cent less than the value for a single unit. Arrangements A, B, and C sedimented head down, while D, E, and F followed a path perpendicular to the longest axis. G and H assumed intermediate and apparently unstable positions during sedimentation.

DISCUSSION

The results of the studies on pH stability of the bacteriophage were similar to those with other viruses in revealing an approximate parallelism between the range of stability of virus activity and the physical stability of the virus particle. As in the instance of other viruses, the range of stability of infectivity was somewhat narrower than that of physical stability. The results of studies being made on the electrophoretic behavior² of the bacteriophage indicate an isoelectric point at about pH 4.2, which corresponds to the acid region where precipitation of the bacteriophage begins. As in the instances of the vaccinia (7), equine encephalomyelitis (8), and influenza (9) viruses, there was no region of redispersion and stability of infectivity below the isoelectric point. This is in contrast to the behavior of the papilloma virus (10).

The "sedimentation constant" of the bacteriophage does not remain a constant with respect to concentration of the phage at either of the two levels observed. This variation is not unique, for it has been observed repeatedly (11, 12) with other purified viruses, the sedimentation constants of which tend, in general, to increase slowly and linearly with dilution to the lowest concentrations with which results can be obtained. A significant difference between the results with the bacteriophage and those with other viruses was seen, however, in the abrupt decrease in the sedimentation constant in the lowest concentration. An explanation of this behavior of the bacteriophage is not apparent. The difference in slopes of the two lines of Fig. 6 in their straight parts may be indicative of differences in aggregation, interaction, and orientation of the phage particles in the ultracentrifuge cell at the different pH values. It is not known whether other viruses show this anomalous behavior at very low concentrations, for their sedimentation rates have not been accurately measured under these conditions. Only the exceedingly great ultraviolet light-absorbing power of the bacteriophage makes its sedimenting boundary visible at concentrations as low as 13 γ per ml. It has been customary to take as the true sedimentation constant the value obtained from extrapolation to zero concentration. A statement of a single sedimentation constant characteristic of the T₂ bacteriophage at infinite dilution is clearly impossible, as the usual methods of extrapolation do not apply.

The extent to which the experiments with lucite models can be employed in explaining the phenomenon of the two sedimentation rates of the bacteriophage remains uncertain. It is clear that the sedimentation of such large models at comparatively high speeds is not influenced by the violent

² Cooper, G. R., Sharp, D. G., Taylor, A. R., Hook, A. E., and Beard, J. W., to be reported.

Brownian movement and other effects to which virus particles are subject while sedimenting in an ultracentrifuge. Nevertheless, the similarity of the behavior of the lucite models in different combinations to that of the bacteriophage in relation to salts or pH seems too close to be entirely fortuitous. It is apparent from the data of Fig. 7, as might have been deduced from the theories of hydrodynamics, that two general types of orientation in fall or sedimentation were encountered in the experiments with the models, and that these were associated with and responsible for the two levels of sedimentation rate observed. The average of the values of relative sedimentation rates of *A*, *B*, and *C* was 96, and that of *D*, *E*, *F*, and *G* was 70. The ratio of the two groups, $96/70 = 1.4$, was remarkably similar to the ratio of the two sedimentation constants of the bacteriophage $1022/695 = 1.5$ (taken from the graph of Fig. 6 at virus concentration of 1 mg. per ml. and pH 4.6 and 6.6, respectively). One might say that if the virus particles were as well oriented as the lucite models, it has been shown that simple aggregation, brought about in different ways, may cause the observed dual sedimentation behavior of the intact bacteriophage. It is difficult to conceive the nature of factors which might be responsible for the aggregation of two or more units under some conditions with head pieces all in the same direction and under others with head pieces and tails in opposite directions. On the other hand, however, if the findings with the lucite models are considered as indications of the behavior of the phage particles, a simple explanation of the two boundaries is at once apparent on the assumption that $S_{20}^0 = \text{about } 1000 \times 10^{-13}$ represents the phage particles dispersed as units and that $S_{20}^0 = \text{about } 700 \times 10^{-13}$ indicates a state of heterogeneous aggregation. Either of these states might readily occur. Some support for the assumption is at hand, for CaCl_2 has been useful in the dispersion, not only of the bacteriophage, but of the equine encephalomyelitis (13) and influenza (14) viruses to facilitate electron micrography. In addition, electron micrographs of the bacteriophage from distilled water reveal the presence of the particles in heterogeneous aggregates.

It is difficult to predict the possible ratio of sedimentation rates of various groups of these lucite models if they were subject to complete disorientation during the process of sedimentation. Qualitatively, however, it seems reasonable to predict a ratio considerably less than the observed one. Experiments designed to study the degree of orientation of the bacteriophage in sedimentation runs are in progress, and the results will be published later.

A further deduction may be made from these experiments; namely, that boundaries resulting from dynamic equilibrium between the two possible states of dispersion mentioned above do not seem to occur, for they would be

seen in the region between $S_{20} = \text{about } 700 \times 10^{-13}$ and $S_{20} = \text{about } 1000 \times 10^{-13}$, where none has been observed. Such boundaries would be expected to vary continuously in sedimentation rate from $S_{20} = \text{about } 700 \times 10^{-13}$ to $S_{20} = \text{about } 1000 \times 10^{-13}$ with changes in the environment. As noted above, no discontinuity in the level of infectivity of the phage was seen at pH 5.8 with the transition of $S_{20} = \text{about } 1000 \times 10^{-13}$ to $S_{20} = \text{about } 700 \times 10^{-13}$. It is not possible, however, to use this finding for interpretations relative to hypothetical states of aggregation. Measurements of infectivity (enumeration of lytic units) were made in high dilutions of the phage in which the concentration of the agent was far below that employed in the ultracentrifugal studies. In addition, the counts of lytic units were made on the host organism in media of about pH 6.5 to 7.0, where only the boundary $S_{20} = \text{about } 700 \times 10^{-13}$ was evident under the conditions of titration.

SUMMARY

The pH range of stability of bacteriophage T₂ infectivity extended from pH 5 to 9. Physical stability of the dispersed particles, determined in sedimentation diagrams, extended through the region of pH 4.6 to 9.5. Below pH 4.5, the phage precipitated but the particle remained intact as low as pH 3.6, as determined by studies on material redispersed at pH 6.6. At pH 10.4, two components were seen, $S = 448 \times 10^{-13}$ and $S = 13.4 \times 10^{-13}$ (not corrected for viscosity or density of the suspending medium).

Two sedimentation constants were observed individually in relation to pH: below pH 5.8 the single sharp boundary had a constant of $S_{20} = \text{about } 1000 \times 10^{-13}$, and above this level there was a single sharp boundary of $S_{20} = \text{about } 700 \times 10^{-13}$. At pH 5.8, both boundaries were seen together, the slower sedimenting component being predominant.

At pH 4.6, the sedimentation constant $S_{20} = \text{about } 1000 \times 10^{-13}$ was linearly related to bacteriophage concentrations from 0.125 to 3.7 mg. per ml. A sharp decrease in the constant occurred at the concentration of 0.041 mg. per ml. Similar relations at the level of $S_{20} = \text{about } 700 \times 10^{-13}$ were seen at pH 6.6, where the sharp decrease in the rate relative to concentration occurred at 0.013 mg. of bacteriophage per ml.

A study of lucite models constructed on the lathe in the shape and proportions of the bacteriophage estimated from electron micrographs showed that the rate of sedimentation of single units and combinations of the units in salt solution was dependent chiefly on orientation in fall. One unit and combinations of more than one unit arranged with tail pieces in the same direction fell at closely similar rates, while the rates of combinations with tails in opposite directions occurred at a different level. The ratio of the two levels of rates of fall of the lucite models was exceedingly

close to that of the two levels of sedimentation rate of the bacteriophage related to pH or to the presence of CaCl_2 reported in previous work.

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CHEMICAL ANALYSIS OF THE T₂ BACTERIOPHAGE AND ITS HOST, *ESCHERICHIA COLI* (STRAIN B)*

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A particulate component of characteristic morphology and behaving biologically as the T₂ bacteriophage of *Escherichia coli* (strain B) has been isolated by centrifugation (1) from lysates of the host grown in nutrient broth and in synthetic saline medium. The purified bacteriophage concentrates exhibited a high degree of homogeneity with respect to particle kind in electron micrographs (1, 2), and of uniformity in size, shape, and density of the particles in ultracentrifugal sedimentation diagrams (1, 3). No material extraneous to the particles of characteristic form was evident in significant amounts by either method of examination.

Previous work by other investigators (4-8) on concentrates of various bacteriophages has revealed the presence of protein, carbohydrate, and nucleic acid, the latter in high concentration. Schlesinger (4) demonstrated an undetermined amount of presumably lipid material in concentrates of a phage of *Bacillus coli* and Pollard (7), corroborating the findings of Schlesinger with respect to protein and nucleic acid, found, in addition, much carbohydrate. Preliminary chemical analyses (1) have shown that the T₂ bacteriophage of *Escherichia coli* is a complex of protein, lipid, and a large amount of nucleic acid, findings contrary to those of Kalmanson and Bronfenbrenner (9), who demonstrated only traces (0.07 per cent) of phosphorus. In the work reported here, the chemical analysis of the T₂ bacteriophage has been extended in a more detailed study of the elementary and component constitution of the agent obtained from both broth and synthetic medium lysates. In addition, it has seemed likely that a direct comparison of the chemical nature of the infectious entity, itself, with that of its single cell host organism, might reveal information providing some insight into the host-virus relationship. Consequently, analyses have been made in parallel on washed concentrates of the host organism, *Escherichia coli* (strain B). The present paper¹ is concerned with a description of the results obtained.

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¹ The results reported here were described before the Division of Biological Chem-

Materials and Methods

The derivation of the bacteriophage analyzed in the present work has been described (1); much of the material was from the same preparations as those employed for ultracentrifugal (1, 3) and electron micrographic examinations (1, 2).

Concentrates of *Escherichia coli* were isolated from cultures in broth and in synthetic medium (1) by centrifugal procedures (1, 10) comparable to those used for purifying the bacteriophage. The cultures were prepared in batches of 15 to 18 liters distributed in 1500 ml. volumes in 2 liter Florence flasks. The seed inoculum of bacteria for the large volumes was prepared in 50 ml. of the respective media in 125 ml. Erlenmeyer flasks, which were inoculated with 0.1 ml. of an 18 to 24 hour broth culture of *Escherichia coli* and then incubated for 18 hours at 37°. To each 1500 ml. volume of medium, previously warmed to 37°, there were added 30.0 ml. of the seed inoculum. The flasks were incubated for 8 hours at 37° with vigorous mixing, manually, every 15 minutes. Stained preparations from the individual 1500 ml. cultures were examined² microscopically at the end of 8 hours, the cultures were pooled, and isolation of the bacteria was begun immediately. The initial sedimentation of the bacteria was carried out in the standard 160 ml. separator bowl of the Sharples centrifuge, rotating 40,000 R.P.M. (39,000*g* at the bowl periphery). The culture fluid was passed through at the rate of 14 liters per hour and was followed by 2 liters of chilled, sterile 0.9 per cent NaCl solution to remove the final 160 ml. of culture medium. The bowl was plugged (10) before rotation ceased, removed from the centrifuge, and shaken vigorously for 15 to 20 minutes to resuspend the bacteria in the 160 ml. of saline solution. The concentrated bacterial suspension was washed into a sterile beaker with 20 to 30 ml. portions of sterile 0.9 per cent NaCl solution, with aseptic (with respect to extraneous organisms) precautions. The suspension was diluted to 400 ml., transferred to 100 ml. lusteroid tubes, and cleared of gross aggregates and a small quantity of flocculated, brown pigmented material by spinning in the horizontal centrifuge (International centrifuge No. 2) at 1000*g* for 5 minutes. The milky white suspension was siphoned into sterile 100 ml. lusteroid tubes and completely sedimented in 30 minutes at 3000*g* in the angle centrifuge. The clear supernatant fluid was poured off and discarded. The large white putty-like pellets were resuspended in 200 ml.

istry at the meeting of the American Chemical Society at Atlantic City, New Jersey, April 8-12, 1946.

² The samples of *Escherichia coli* analyzed were obtained during the course of the studies on bacteriophage. There was never any evidence, microscopic or cultural, of contamination with other organisms.

of sterile saline with the aid of glass beads. The sequence of horizontal and angle centrifugation was repeated three additional times. After the final sedimentation, the pellets were suspended in a volume of 50 to 75 ml. of sterile 0.9 per cent NaCl solution. All transfers were made under ultra-violet light (10), and all centrifugation was carried out with the material in closed sterile tubes.

Qualitative Analysis—The qualitative chemical findings with concentrates of the bacteriophage from both broth and synthetic medium cultures have already been reported (1). The usual protein tests applied to the bacterial concentrates, biuret, ninhydrin, Millon's, and xanthoproteic, were positive. The Molisch reaction was immediately positive and became progressively stronger with standing of the mixture. The Sakaguchi test was negative, and the Ehrlich benzaldehyde test for tryptophane was positive. A strongly positive pentose reaction was obtained with Bial's reagent (orcinol-HCl-FeCl₃). Both the Schiff and diphenylamine tests were positive. A green color was obtained on heating 10 minutes in the water bath at 100° with tryptophane and perchloric acid (11). Spectrophotometric examination of an isoamyl alcohol extract of the chromogenic material revealed a curve characteristic of ribopentose (sodium thymonucleate and yeast nucleic acids were used as controls). This is in direct contrast to the result of the comparable experiment (1) with bacteriophage in which a red chromogenic material showed a curve characteristic of desoxypentose.

Quantitative Analysis—Concentrates obtained from large pools of starting material, as described above, and containing 100 to 200 mg. of bacteriophage from each type of medium and 0.5 to 2 gm. of bacteria from the respective media were dialyzed against running distilled water at 2-8° for 72 to 96 hours, frozen with dry ice, and lyophilized. The bacteriophage and bacterial preparations were treated and analyzed in identical ways. The results of elementary and component analyses of the four materials and fractions are given in Tables I and II.

Analyses for carbon, nitrogen, phosphorus, and carbohydrate were made on the whole bacteriophage complex and on the unfractionated bacterium. Samples of 4 to 7 mg. of the frozen and dried materials were weighed into small Pyrex boats. After the samples were dried to constant weight at 40-45° over P₂O₅, the weights were taken in closed weighing bottles. Carbon was determined by wet combustion (12), nitrogen by the semimicro-Kjeldahl method with the digestion mixture described by Kirk, Page, and Van Slyke (13), phosphorus by the method of Kirk (14), and carbohydrate, as glucose, by the method of Tillmans and Philippi (15).

Fractionation with Lipide Solvents—Two samples of 100 to 150 mg. each of the whole bacteriophage or bacterial complex were dried to constant weight, as described above. The samples were transferred to 40 ml.

extraction tubes provided with ground glass connections and extracted with alcohol-ether, 3:1, for 2 hours under a reflux condenser. Details of the manner of the transfer of the alcohol-ether-insoluble material to a

TABLE I
Elementary and Fractional Analyses of T₂ Bacteriophage and Its Host, Escherichia coli (Strain B)*

		Broth bacteriophage		Synthetic medium bacteriophage		Broth <i>E. coli</i> (8 hr. culture)		Synthetic medium <i>E. coli</i> (8 hr. culture)	
		Whole complex	Fraction	Whole complex	Fraction	Whole complex	Fraction	Whole complex	Fraction
Whole complex	Carbon	42.0		42.3		49.1		49.0	
	Nitrogen	13.5		13.3		13.2		13.2	
	Phosphorus	4.84		5.22		2.72		2.66	
	Carbohydrate†	13.6		11.7		12.5		11.6	
Alcohol-ether-soluble fraction	By difference	6.3		1.72		7.98		8.85	
	Carbon	2.56	40.6	1.36	79.0	6.00	75.3	5.72	64.7
	Nitrogen	0.19	3.0	0.10		0.16		0.17	
	Carbohydrate†	0.23	3.7	0.05		0.13		0	
	Non-lipide	3.70	58.8			0.23			
Petroleum ether-soluble fraction	Total lipide‡	2.61		1.77		7.61		8.83	
	Carbon	2.00	76.6	1.36	76.8	5.07	65.5	5.89	66.7
	Nitrogen	0		0		0.17		0.16	
Alcohol-ether-insoluble fraction	Phosphorus	0		0		0.33		0.39	
	By weight	93.7		98.3		92.0		91.2	
	Carbon	39.6	42.3	41.4	42.1	42.9	46.7	43.0	47.3
	Nitrogen	13.2	14.1	13.3	13.5	13.1	14.2	13.0	14.3
	Phosphorus	4.86	5.20	5.20	5.29	2.40	2.61	2.22	2.44
	DNA§ P	3.99	4.26	4.41	4.49	0.52	0.57	0.24	0.26
	RNA§ "	0.62	0.65	0.12	0.13	1.81	1.97	1.97	2.16
	Other "	0.26	0.27	0.66	0.67	0.07	0.08	0.02	0.02+
	Carbohydrate†	13.1	14.0	11.2	11.3	11.2	12.2	11.8	13.0

* Each of these values represents the percentage of dry weight of the whole complex or fraction.

† The carbohydrate values are expressed as glucose.

‡ The total lipide values of the bacteriophage = carbon values \times 1.3; those of the bacterium = carbon values \times 1.5.

§ DNA designates desoxypentose nucleic acid; RNA designates ribopentose nucleic acid.

tared, sintered glass filter and the collection of the alcohol-ether fraction in a 50 ml. volumetric flask have been described (16). The alcohol-ether-insoluble fractions were dried to constant weight. Each of the respective values in percentage by weight shown in Table I is the average of two independent weights (of the two samples), which in all cases showed an

agreement of 1 per cent or better. The percentage of alcohol-ether-soluble material was found by difference. Alcohol-ether-soluble carbon and nitrogen were determined (13) in 2.0 and 5.0 ml. aliquots and carbohydrate was determined (15) in 10.0 ml. aliquots after evaporation to dryness. Aliquots of 5.0 ml. were examined (13) for total cholesterol.

To obtain the total lipid values, a 25 ml. aliquot was withdrawn from each of the duplicate alcohol-ether fractions. The two aliquots were combined in a beaker and evaporated to dryness at 58°. The residue was

TABLE II
Component Constitution* of *T₂* Bacteriophage and Its Host, *Escherichia coli* (Strain B)

	Lipide				Non-lipide				
	Total	Phospholipide	Cholesterol	Neutral fat	Total	Protein	Carbohydrate†	Nucleic acid‡	
								DNA	RNA
Broth bacteriophage..	2.61§	0	0	2.61	97.4	50.6	13.6	40.3	6.6
Synthetic medium bacteriophage.....	1.77§	0	0	1.77	98.2	52.4	11.7	44.6	1.3
Broth <i>E. coli</i>	7.75	7.75	0	0	92.3	67.9	12.5	5.2	19.1
Synthetic medium <i>E. coli</i>	9.11	9.11	0	0	90.9	67.7	11.6	2.4	20.9

* Each of these values represents percentage of dry weight of the whole complex.

† Expressed in terms of glucose.

‡ DNA = desoxypentose nucleic acid; RNA = ribopentose nucleic acid. To obtain the nucleic acid values, the DNA phosphorus values of the alcohol-ether-insoluble fraction were multiplied by 10.1; the RNA phosphorus values were multiplied by 10.6.

§ The total lipid values for the bacteriophage were calculated by multiplying the carbon values given in Table I by the factor 1.3.

|| The total lipid values of the bacterium were calculated as phosphatide from the phosphorus content of the petroleum ether-soluble fractions given in Table I.

extracted with five 5 ml. portions of redistilled petroleum ether which had been washed with concentrated H₂SO₄. The petroleum ether extracts were filtered into a 25 ml. volumetric flask. Carbon, nitrogen, and phosphorus were determined (13) in 2.0 and 5.0 ml. aliquots. Total lipid values (Table I) for the bacteriophage preparations were calculated from the carbon values of the petroleum ether-soluble fraction, with the factor 1.3 (the factor for neutral fat). The analogous total lipid values for the bacterium were obtained by multiplying the carbon value by 1.5 (the factor for phospholipide).

Alcohol-Ether-Insoluble Fraction—Samples of the non-lipide fraction were dried to constant weight for the determination of carbon, nitrogen,

and carbohydrate, as described for the samples of whole complex. For the determination of the total alcohol-ether-insoluble phosphorus and fractionation into DNA phosphorus (desoxypentose nucleic acid phosphorus), RNA phosphorus (ribopentose nucleic acid phosphorus), and inorganic or protein phosphorus (designated as "other" phosphorus in Table I), a modified micro technique based upon the method of Schmidt and Thannhauser (17) was employed. The alcohol-ether-insoluble fractions of the bacteriophage and the bacterium were analogous to Schmidt and Thannhauser's extracted tissue powder. Samples of the bacteriophage fractions weighing 15 to 18 mg. (constant dry weight) and of bacteria weighing 25 to 30 mg. were placed in 20.0 ml. volumetric flasks with 15 ml. of 5 per cent KOH solution. The flask, closed with a glass stopper, was incubated at 37° for 15 to 18 hours. The material was completely dissolved at the end of this period. The flask and contents were cooled to room temperature, and the volume was made up to 20 ml. A 2 ml. aliquot of this solution was taken for the total phosphorus determination by the method of Kirk (14). A 15 ml. aliquot was transferred to a tube graduated at 20 ml. and the desoxypentose nucleic acid precipitated by the addition of 3.0 ml. of 6 N HCl and 2.0 ml. of 20 per cent trichloroacetic acid. The mixture, chilled in an ice bath and stirred frequently during a 15 minute period of standing, yielded a well flocculated precipitate. The latter was removed by filtration (Whatman paper No. 50, with very gentle suction) and the total acid-soluble organic and inorganic phosphorus determined in a 5.0 ml. aliquot of the filtrate. The inorganic or other phosphorus was precipitated according to Delory (18) from a 10.0 ml. aliquot of the filtrate. The respective phosphorus values (Table I) and the nucleic acid values shown in Table II were calculated as described by Schmidt and Thannhauser (17).

In view of the low content of ribopentose nucleic acid of the synthetic medium bacteriophage, as shown in Table II, the phosphorus fractionation of this material was repeated with another 20 mg. sample. The results were 5.10 per cent total alcohol-ether-insoluble phosphorus, 4.40 per cent DNA phosphorus, 0.13 per cent RNA phosphorus, and 0.54 per cent other phosphorus, values corroborating those of Table I. As a further control experiment, a mixture containing known amounts of DNA, RNA, and casein was analyzed in the manner described for the bacteriophage preparations. The mixture contained 1.02, 0.26, and 0.09 mg. of DNA, RNA, and casein phosphorus, respectively; the amounts found on analysis were 0.98 mg. of DNA, 0.25 mg. of RNA, and 0.1 mg. of casein phosphorus.

Determinations of the nucleic acid content of the bacterium and the bacteriophage from broth were made also by means of the perchloric acid-tryptophane method of Cohen (11). The value, 19.1 per cent, obtained for the bacterium was identical with the amount of *ribopentose* nucleic acid

determined by the method of Schmidt and Thannhauser. In the instance of the bacteriophage, the value was 42.0 per cent, which was slightly higher than the amount of *desoxyribose* nucleic acid found by the other method.

The determined or calculated component constitutions of the bacteriophage isolated from the two media and of the corresponding preparations of bacteria are given in Table II. The total lipid values for the phage preparations were calculated from the carbon values of the petroleum ether-soluble fractions, while the total lipid values for the bacterial preparations were calculated from the petroleum ether-soluble phosphorus values. Total non-lipid is expressed as the difference between the whole complex and the total lipid value. The protein value represents the total non-

TABLE III

Apparent Partial Specific Volume of T₂ Bacteriophage of Escherichia coli (Strain B)

Broth Bacteriophage 33			Broth Bacteriophage 34			Synthetic Bacteriophage 39		
Ultra-centrifugal cycle*	Concentration of phage	Apparent partial specific volume	Ultra-centrifugal cycle*	Concentration of phage	Apparent partial specific volume	Ultra-centrifugal cycle*	Concentration of phage	Apparent partial specific volume
	mg per ml			mg per ml			mg per ml	
1	7.94	0.6542	1	7.33	0.6588	1	5.08	0.6693
1	7.94	0.6551	1	7.33	0.6589	1	5.08	0.6687
1	7.94	0.6538				1	5.08	0.6709
2	7.47	0.6542	2	6.63	0.6584			
2	7.47	0.6559	2	6.63	0.6544			
2	7.47	0.6533	2	6.63	0.6553			

* The number of times the material was sedimented in the vacuum type ultracentrifuge; each preparation had been sedimented one additional time (the initial preliminary concentration) in the Sharples centrifuge.

lipide value minus the total nucleic acids. The carbohydrate values are those of the respective whole complexes.

Apparent Partial Specific Volume of T₂ Bacteriophage—For the estimation of partial specific volumes, concentrates containing 5.0 to 8.0 mg. of bacteriophage per ml. of suspension in a solution composed of 0.9 per cent NaCl and 0.1 per cent KCl (3) were analyzed by the method described in detail for the influenza viruses (19). The partial specific volume was calculated by means of the equation given by Kraemer (20). The values obtained for the apparent partial specific volume of two individual batches of T₂ bacteriophage from broth lysates are shown in Table III. After the first ultracentrifugal cycle, replicate determinations were made; the materials were then diluted to 120 ml., angle centrifuged, and again ultracentrifuged. The replicate determinations on the material after the second ultracentrifugation were virtually identical with those obtained after the

first ultracentrifugation. Partial specific volume determinations on the synthetic medium bacteriophage were made on material ultracentrifuged only once, owing to the instability of the agent. The replicate values obtained with a representative batch of bacteriophage from synthetic medium are shown in Table III. The reciprocal of the average apparent partial specific volume of the broth bacteriophage indicates an approximate dry density of 1.52, which is in accord with the chemical composition of the material.

DISCUSSION

The ultracentrifugal and electron micrographic evidence of the physical homogeneity of the bacteriophage concentrates selected for the present study has already been described (1-3). An index of the chemical homogeneity is seen in the constancy of the values of apparent partial specific volume, Table III, which indicates a chemical and physical entity of uniform composition. These results with the bacteriophage were similar in this respect to those with the influenza viruses (19), which are entities of constant composition having a limiting partial specific volume characteristic for each strain.

The apparent partial specific volume of the bacteriophage was in accord with its chemical composition, and the level of the values for the broth bacteriophage did not change on repeated ultracentrifugation. Though instability (1) attending repeated ultracentrifugation precluded a like study on the agent from synthetic medium, this material nevertheless gave values after a single ultracentrifugation closely similar to those observed for the broth bacteriophage.

The results of the chemical examinations given in Tables I and II show that the T₂ bacteriophage consists of protein, a small proportion of lipid, and a very high content of nucleic acid. The nucleic acid, Table II, consisted of both desoxypentose and ribopentose types, the former in a considerably larger amount than the latter. Significant differences between the bacteriophage derived from broth medium lysates and that from synthetic medium lysates were evident in the distribution of the two types of nucleic acid (Table II), as determined in the alcohol-ether-insoluble fraction (Table I) by the phosphorus fractionation method of Schmidt and Thannhauser (17). Attempts to dissociate the nucleic acid and protein directly in the whole complex suspended in NaCl solution by the method of Hammersten (21) were unsuccessful; the bacteriophage complex remained intact. Though the total amount of nucleic acid of the broth phage, determined on the basis of phosphorus, was essentially the same as that of the synthetic medium phage, the content of the ribopentose type of the former was five times as great as that of the latter. Another significant

difference, Tables I and II, between the broth bacteriophage and that from synthetic medium was in the content of lipide.

Extraction of the broth bacteriophage with alcohol-ether resulted in solution of 6.3 per cent (Table I) of the weight of the complex. Of this amount, slightly less than half, 2.61 per cent of the whole complex, was soluble in petroleum ether in subsequent extraction. The nature of the major portion of the non-lipide material soluble in alcohol-ether, 3.7 per cent of the whole complex, was not established; a small proportion, 3.7 per cent of the alcohol-ether-soluble fraction, reacted with orcinol-sulfuric acid. In the instance of the synthetic medium phage, the material extracted from the complex by alcohol-ether was essentially all lipide, completely soluble in petroleum ether.

The total lipide content of the bacteriophage, Table I, was considerably lower than the 10.4 per cent of extractable lipide reported for a *Bacillus coli* phage studied by Pollard (7) and McIntosh and Selbie (8, 22). The methods used for extraction were not described in detail. Schlesinger (4) recorded the finding of ether-extractable lipide in a *Bacillus coli* phage but gave no values. This author also stated that after acid hydrolysis considerably larger quantities of ether-soluble material, in excess of that directly soluble in ether, were obtained. In the course of the present work, samples of the alcohol-ether-insoluble fractions of the bacteriophage were examined for firmly bound lipide by reextracting with alcohol-ether, 1:1, acidified with hydrochloric acid (0.1 N total acid concentration). The acid alcohol-ether extracts contained no petroleum ether-soluble lipide but did contain 10 to 12 per cent (of the dry weight of the whole complex) of nitrogenous bases, apparently split off from the nucleic acids. No phosphorus was extracted by the acid alcohol-ether.

In the lipide or petroleum ether fraction of the bacteriophage, there was no nitrogen or phosphorus and, therefore, no phospholipide. Cholesterol was, likewise, absent, and the lipide was calculated as neutral fat. As seen in Table II, the amount of lipide in the broth bacteriophage was significantly greater, 35 per cent, than that in the phage from synthetic medium.

The alcohol-ether-insoluble fractions, Table I, contained the protein and nucleic acids. The weight percentages agreed well with the total lipide found in the petroleum ether-soluble fraction of the synthetic medium phage, but not in the case of the broth bacteriophage, as noted above. The principal differences between the broth and the synthetic medium phage are seen in the total alcohol-ether-insoluble phosphorus values and the distribution of this phosphorus into DNA, RNA, and "other" phosphorus. Phosphorus other than that in nucleic acid, presumably inorganic or associated with protein, was present in both broth and synthetic medium phage, considerably more in the latter than in the former. It might be considered

that this represents inorganic phosphorus carried over in the purification process from the synthetic medium. This is questionable, since the bacteria grown in synthetic medium contain the least amount of other phosphorus, 0.02 per cent, as shown in Table I. There may exist, however, differences in the adsorptive properties of the two materials (bacteriophage and bacterium) for phosphate. The carbohydrate content of the alcohol-ether-insoluble fraction of the bacteriophage was similar to that of the whole complex, Table I, confirming the absence of appreciable amounts of carbohydrate in the alcohol-ether-soluble material.

It is of interest to note that the total carbohydrate content of the bacteriophage is approximately one-half that to be expected if all of the carbohydrate present in the whole complex is associated with the nucleic acids. With orcinol and sulfuric acid, the pyrimidine nucleotides and nucleosides are hydrolyzed incompletely (23). It would thus appear that no carbohydrate is present in the bacteriophage other than that associated with the nucleic acids.

The findings obtained with the bacterium, given also in Tables I and II, show that the *Escherichia coli* organism, too, is a complex of protein, nucleic acid, and lipide. The nitrogen content of the whole bacterial complex was about the same as that of the phage, but the carbon content was considerably greater, related probably to the higher lipide content, Tables I and II. The carbohydrate content of the bacterium was approximately the same as that of the phage, but, as noted below, probably differed with respect to constitutional relations within the bacterium.

The material of the bacterium soluble in alcohol-ether was soluble likewise in petroleum ether, as shown by the close agreement of the findings, Table I, with these two fractions. In contrast with that of the bacteriophage, the lipide of the bacterium contained nitrogen and phosphorus. The atomic ratio of the elements was 1:1, indicating a lecithin type of phospholipide. Neither neutral fat, found in the phage, nor cholesterol was present. In view of the difference between the bacteriophage and the bacterium, it would be difficult to consider either the neutral fat or the phospholipide as a contaminant carried over in the process of purification. The total lipide content of the bacterium was considerably greater than the lipide content of the phage, and slightly more was seen in the bacterium grown in broth than that in synthetic medium. The total bacterial lipide observed here was much higher than the 1.6 per cent of extractable lipide found by Pollard (7). Leach (24), Dawson (25), and others have shown that the age, conditions of growth, and especially the composition of the culture medium markedly influence the composition of *Escherichia coli*.

The nucleic acid of the bacterium, approximately half the amount found in the bacteriophage, also consisted of both desoxypentose and ribopentose

types, as shown by phosphorus fractionation of the alcohol-ether-insoluble fraction. In the bacterium, however, the ribopentose type was predominant. Schaffer, Folkoff, and Bayne-Jones (26) isolated a nucleic acid preparation from *Bacillus coli* which contained approximately the amount of phosphorus required for a ribopentose nucleic acid. Guanine was found by these authors, but the pentose reaction was reported to be negative. As in the case of the bacteriophage, the ratio of the two types of nucleic acid varied in relation to the medium used, but in contrast with the synthetic medium phage, the desoxypentose nucleic acid was less in amount in the synthetic medium bacterium. The carbohydrate of the alcohol-ether-insoluble material agreed closely with the values for the whole complex. In contrast with the phage, the amount of carbohydrate of the bacterium, 12 per cent, was greater than could have been bound in the nucleic acid present. Since complete recovery of all of the carbohydrate of the nucleic acid was unlikely (23), and judging from the proportion of carbohydrate to nucleic acid found in the bacteriophage, it appears probable that the bacterium contains significant amounts of carbohydrate not associated with nucleic acid.

In so far as comparisons can be made, the constitution of the T_2 bacteriophage of *Escherichia coli* appears similar, qualitatively, to the agents studied by Schlesinger (4), Pollard (7), and McIntosh and Selbie (8). Certain quantitative differences were evident, but it should be borne in mind that the agents studied by these authors may not have been identical with the strain, T_2 , examined here. It can scarcely be assumed that all bacteriophages of *Escherichia coli* will have the same constitution and, consequently, a detailed comparison of quantitative differences would not be significant. Cohen,³ however, in a study of the T_2 bacteriophage, has found a smaller amount of phosphorus, 3.8 per cent, than the amounts, 4.84 and 5.22 per cent, found here. The differences may well be related to the type of medium, sodium lactate (Friedlein), used by Anderson (27), who furnished the material analyzed by Cohen,³ and the broth and the sodium lactate-free NH_4Cl media employed in the present work. Cohen³ found only desoxypentose nucleic acid with the perchloric acid-tryptophane method (11), in an amount which was in agreement with the 3.8 per cent of phosphorus. With this method (11), it was not possible in the present work to demonstrate quantitatively small amounts of one type of nucleic acid in the presence of relatively large quantities of the other type. The results found in the present studies are almost entirely at variance with those on the T_2 phage obtained by Kalmanson and Bronfenbrenner (9). These authors found only 0.07 per cent of phosphorus in T_2 bacteriophage

³ Cohen, S. S., personal communication.

isolated from sodium lactate medium by means of ultrafiltration, and the nitrogen values were too variable for judgment of their significance. The elementary analyses of the T₂ bacteriophage reported here were similar to the results obtained by Northrop (6) with a staphylococcus bacteriophage, with the exception of the carbohydrate value of 1.5 per cent.

The T₂ bacteriophage exhibits a morphological and constitutional complexity resembling that of the animal viruses more closely than that of the corresponding agents of plant diseases, except in the high total content of nucleic acid, which is comparable to the amount found in tobacco ring spot virus (28). The presence of both desoxypentose and ribopentose nucleic acids also constitutes a difference from the animal viruses previously studied; in the vaccinia (29) and rabbit papilloma (30) viruses, only desoxypentose nucleic acid has been demonstrated, and in the agent of equine encephalomyelitis (31)⁴ only the ribopentose type. Knight (32) has reported the presence of the ribopentose type in the influenza virus, though only the desoxypentose type has been found in work (16) in this laboratory. Ribopentose nucleic acid is the type found in plant viruses. The absence of phospholipide and cholesterol constitutes a unique difference between the lipid constitution of the bacteriophage and that of the animal viruses. While the bacteriophage contains only neutral fat, in all other cases in which lipid has been found associated as an integral part of a virus complex, phospholipide, cholesterol, and neutral fat have all been present (16, 29, 31, 32),⁴ usually in definite proportions characteristic of the virus or its strain.

The qualitative constitutional similarities of the bacteriophage and the host organism, *Escherichia coli*, are clearly shown in Tables I and II. An outstanding difference was observed in the kind of lipid associated with the two materials. The atomic ratio, 1:1, of lipid nitrogen and phosphorus in the bacterium is that of a phospholipide of the lecithin type which, incidentally, resembles lecithins similar to those found in vaccinia (29) virus and a normal component (33) of chick embryo tissue. This phospholipide differs from the analogous constituent of the influenza (16) and the equine encephalomyelitis (31)⁴ viruses. Quantitatively, wide differences are evident between the structure of the bacteriophage and that of its host. Especially notable are the total lipid contents, the distribution of the two types of nucleic acid, and the proportional contents of carbohydrate.

A remarkable character of the bacteriophage was the definite variation in size as seen in electron micrographs (1, 2) and chemical constitution as described above in relation to the medium in which the host organism was cultured. These findings and others, previously discussed (1), indicate that the bacteriophage behaves as an autonomous entity, responding like a

⁴ Taylor, A. R. Sharp, D. G., Beard, D., and Beard, J. W., unpublished work.

living organism to a changing environment. The bacteriophage increases only in the presence of the host bacterium and to do so apparently enters (34) the host cell. It appears likely that the influence of medium on the composition of the bacteriophage is indirect and dependent on a primary effect of the medium on the bacterium, an effect disclosed in the variation seen in the composition of the bacterial host in relation to the medium. A lack of parallelism between the variation of the bacteriophage and that of the bacterium was seen in the opposite changes in the proportions of the two types of nucleic acid and in the amounts of lipide. It is evident that the processes involved in the formation of the phage and responsible for its specific constitution are to an extent independent, not only of the qualitative aspects of bacterial metabolism and constitution in a given medium, but also of quantitative changes within the bacterium in relation to medium.

SUMMARY

Analyses were made of the chemical constituents of the T_2 bacteriophage of *Escherichia coli* (strain B) obtained from lysates of the host in broth and synthetic medium and of the host bacterium cultured in like media. The bacteriophage consists of protein, lipide, and nucleic acid. The lipide fraction exhibited the behavior of neutral fat, and neither cholesterol nor phospholipide was present. There were present both desoxypentose and ribopentose nucleic acids, of which the former was greatly predominant. The amount of carbohydrate found was approximately half the theoretical amount bound in the nucleic acids and was considered to be wholly associated with the nucleic acids.

The bacterium likewise consisted of protein, lipide, and nucleic acid. In contrast with the bacteriophage, the lipide contained nitrogen and phosphorus. The atomic ratio of these elements indicated a phospholipide of lecithin type. Neutral fat and cholesterol were absent. The nucleic acid consisted, as in the bacteriophage, of the desoxypentose and ribopentose types, but, unlike the ratio of these constituents in the virus, the quantity of the ribopentose type greatly exceeded that of the desoxypentose type. The amount of carbohydrate found was greater than the quantity theoretically associated with the nucleic acids.

The apparent partial specific volume of the bacteriophage from broth lysates was 0.655 and that from synthetic medium lysates was 0.669, values in accord with the chemical constitution.

Variation was seen in the composition of the bacteriophage and in that of the host in relation to the type of medium in which the bacterium was cultured. The quantitative changes in certain of the constituents of the bacteriophage, for example the ratio of the two types of nucleic acid, did not parallel analogous changes in the bacterium.

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THE EFFECT OF FORMALDEHYDE ON THE ACIDIC AND BASIC PROPERTIES OF WOOL

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Previous papers have reported the effects of salt concentration (2) and temperature (10) on the extent of combination of wool with hydrochloric acid and sodium hydroxide. As with other proteins, the tendency of wool to combine with acids and bases is also greatly influenced by the presence of formaldehyde (3). An investigation of this influence is reported in the present paper.¹ Owing to the special methods which are available for studying the combination of wool with base (10), investigation of the equilibria involving wool, alkali, and formaldehyde may extend into the strongly alkaline range without complications caused by the gradual destructive effect of alkali on sulfur-containing proteins.

The pH values of alkaline solutions of proteins or amino acids are considerably decreased by the addition of formaldehyde. Over a wide range of pH, the effect is such as to make it appear that the presence of formaldehyde increases the acid dissociation constants of certain positively charged groups in the protein (principally RNH_3^+ groups from lysine side chains in proteins, or the α -amino groups of amino acids). The dissociations of other groups (with the possible exception of guanidonium groups) appear to be very little affected. This selective action, which has been investigated in great detail (1-3, 15, 16, 21, 22), has been shown to depend on the combination of un-ionized amino groups in amino acids with the formaldehyde. Applications of this reaction to the estimation of amino acids, the measurement of protein hydrolysis, and the estimation of the primary amino groups of various proteins (5, 11-14, 18) are well established.

Procedure

Measurements were made of the amounts of hydrochloric acid and potassium hydroxide combined by wool in the presence of two concentrations of

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¹ This paper is not concerned with the partially irreversible combination of formaldehyde with proteins, which requires high concentrations of aldehyde and relatively long periods of time. This combination is important in the production of

formaldehyde (0.25 and 1.0 M), over the pH range 3.0 to 13.3. The measurements were made at 0°, primarily to minimize decomposition of the wool by alkali, but it is also convenient to work at low temperatures,² because the dismutation of formaldehyde to methanol and formic acid at higher temperatures results in the rapid neutralization of base, and introduces large errors in the determinations of base bound by wool.

Details of the purification of the wool, determination of the acid or base combined, and correction of the results for the effects of decomposition of the wool have been described elsewhere (19, 20). Although formaldehyde is a weak acid (pK greater than 13), there was no need to estimate and correct the base bound by formaldehyde, because the base bound by wool was always determined by comparing the base present at equilibrium in aliquots of two solutions identical except for the fact that one contained wool and one did not.

Stock solutions of formaldehyde were freshly prepared by distilling paraformaldehyde into water; they were assayed by oxidation of the aldehyde with hydrogen peroxide to formic acid, which was titrated (8). The experimental solutions, containing formaldehyde and base, were made up at 0° and never exposed to higher temperatures. With alkaline solutions, 24 hours sufficed for the attainment of equilibrium; with acid solutions, 48 hours.

RESULTS AND DISCUSSION

Effect of Formaldehyde on Titration Curve of Wool

Titration curves of wool, obtained at a constant ionic strength³ (0.2 molal KCl) in the presence and absence of formaldehyde, are shown in Fig. 1. At the lowest pH values shown, the curves tend to come together, but, in more weakly acid solutions, formaldehyde slightly reduces the amounts of acid bound by wool. At higher pH values, the curves become widely separated. Those obtained with formaldehyde present are displaced from the control curve (no formaldehyde) in the direction of more acid pH values. The displacement reaches a maximum of about 1.9 units, when

protein plastics and artificial protein fibers (6, 9) and possibly plays a part in the protection of protein fibers from damage caused by alkali (4).

² Over half of the base added to the 1.0 M formaldehyde solutions at 25° is neutralized by formic acid within 24 hours. At 0°, less than 1 per cent of the base is affected in the same time.

³ The ionic strength was kept constant within narrow limits by the addition of an appropriate amount of potassium chloride to each solution, calculated on the basis of preliminary experiments which permitted estimation of the equilibrium concentration of the base. In this calculation the ionization of formaldehyde was disregarded, since measurements with the glass electrode showed that it made almost negligible contributions to the ionic strength at 0° even at pH 13.

the amounts of base bound are between 0.15 and 0.20 m μ per gm. of wool. The small displacement found in acid solutions depends noticeably on the formaldehyde concentration, whereas, in the alkaline range, almost a maximum effect appears to be produced by the lower of the two concentrations used. These effects on the titration curve differ from those previously reported for soluble proteins only in that they are somewhat smaller

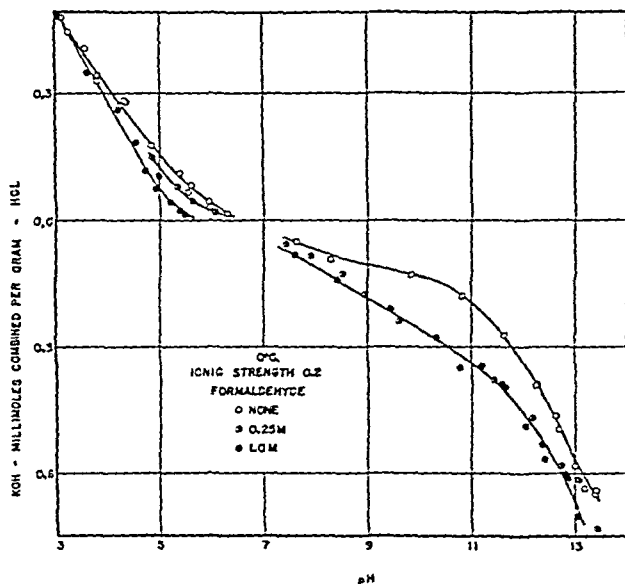


FIG. 1. The effect of formaldehyde on the dependence on pH of the combination of wool with hydrochloric acid and potassium hydroxide at a constant ionic strength (0.2). A separate curve has been drawn for the measurements obtained at the lower concentration of formaldehyde, only for the combination with acid. Measurements in the extremely unbuffered range near neutrality are not represented, and the curves are not defined in this region.

(consistent with the smaller lysine content of wool), and show a lower degree of dependence on the concentration of aldehyde.

Only certain of the dissociating groups in proteins are affected by formaldehyde. When the effect of the formaldehyde is very large, so that the alkaline branch of the titration curve obtained in its presence is displaced by 3 or more pH units from the control curve, a very simple analysis of the effect may be made (12). This simplicity results from the fact that practically the entire range of titration of a single set of dissociating groups (having a given dissociation constant) occurs within a range of 3 pH units. If only one such set of groups is affected by formal-

dehyde, then, in the pH range of the displacement, the base bound by these groups in the presence of formaldehyde will be maximum throughout the entire range of the displacement, and the titration of these groups will make no contribution to the slope of the titration curve in this range of pH.

In such a simple case, and in the same range of pH, merely subtracting the ordinates of the control curve from the corresponding ordinates of the

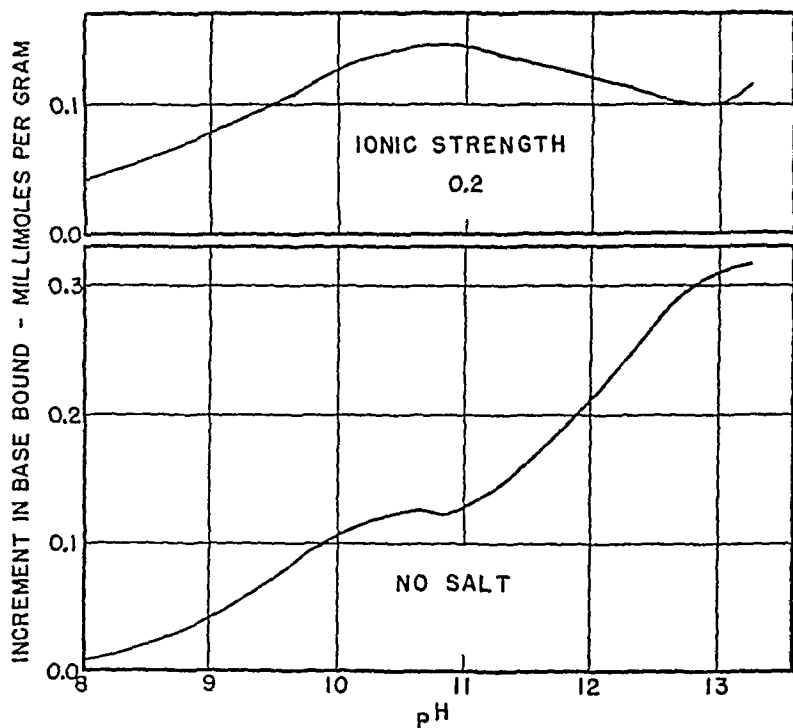


FIG. 2. The increment in base bound by wool brought about by the presence of 1 M formaldehyde.

curve obtained with formaldehyde present will give the characteristic titration curve *in formaldehyde* of the set of dissociating groups which react with formaldehyde (12). The base bound by other groups not affected by formaldehyde will have been eliminated by the subtraction. Actually two curves will be obtained, an ascending S-shape curve just described, followed by a plateau, and then a descending S-shaped curve, which is the *inverted* titration curve of the same set of groups in the absence of formaldehyde. The presence of the plateau is the criterion which determines whether this simple case is actually realized. When no distinct plateau is found, the curve of the groups affected by formaldehyde has been displaced less than 3 to 4 pH units, and the maximum difference in ordinates ob-

tained is less than the total base-binding capacity of the set of groups with which we are now concerned.

An application of this reasoning to the data of Fig. 1 is shown in the upper section of Fig. 2. No distinct plateau is found. If the affected groups are identified with the ϵ -amino groups of lysine, the indicated lysine content of wool is *greater* than 2.2 per cent. The value determined by the amino nitrogen method of Rutherford, Harris, and Smith (17) is 3.3 per cent

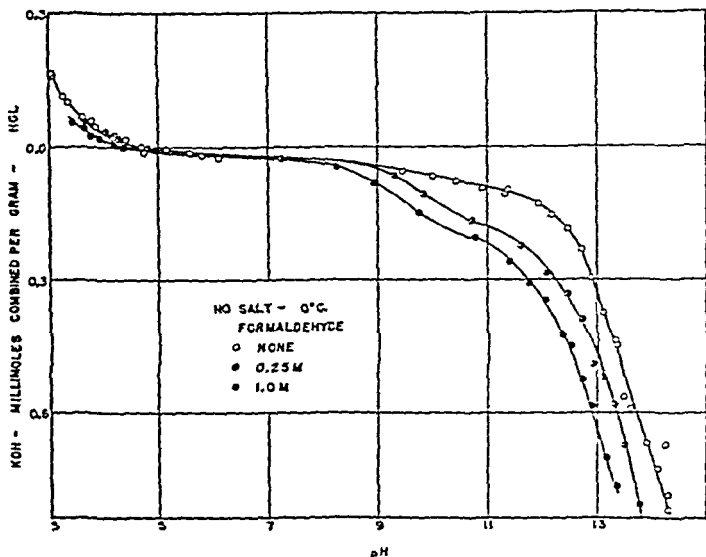


FIG. 3. The effect of formaldehyde on the dependence on pH of the combination of wool with hydrochloric acid and potassium hydroxide, in the absence of added salt.

The titration constant indicated by the pH coordinates of the ascending branch of the curve of differences (Fig. 2) is roughly $10^{-3.7}$. Since the displacement caused by formaldehyde (Fig. 1) is less than 2 pH units, the negative logarithm (pK) of the titration constant of these groups in the absence of formaldehyde is less than 11.7. The value indicated by the work of Kekwick and Cannan on egg albumin (12) is about 11.⁴

The fact that the descending branch of the curve is not fully symmetrical with respect to the ascending branch is discussed below.

Data obtained in the absence of salt are shown in Fig. 3. Here the

⁴ At pH 9.7, base bound by wool in the presence of formaldehyde is about 0.23 mm per gm. Of this amount, the ϵ -amino groups of lysine contribute about 0.12 mm, and histidine accounts for about 0.05 mm. The balance, 0.06 mm, presumably represents acidic groups in tyrosine side chains. Since the total tyrosine content is about 0.32

maximum separation of the curves along the pH axis is somewhat larger than in Fig. 1 (2.35 units instead of 1.90), and differences in the effects of the two concentrations of formaldehyde are much clearer than when salt is present. When the difference curves for these data are plotted as in Fig. 2 (lower section), the lack of symmetry previously noted becomes clearer, and its cause apparent. Instead of one set of groups affected by formaldehyde, two are indicated. One of these (pH values below 11) is apparently the same as that indicated in the experiment with salt, and probably corresponds to the ϵ -amino groups of lysine. The other occurs in a more alkaline range, suggestive of the guanidino groups of arginine. The failure of the latter to manifest themselves strongly in the data obtained with salt present appears to indicate that the combination of formaldehyde with these groups is inhibited by salt. The reason for this inhibition remains obscure. Failure to observe a pH effect of formaldehyde in the arginine groups in other proteins is probably due to the absence of data for these proteins in strongly alkaline solutions. An effect of formaldehyde on the guanidino group of arginine itself is now well known (7, 9).

The amounts of lysine and arginine indicated in the graph have significance only as minimum values. The differences attributed to arginine are somewhat more arbitrary than those attributed to lysine, because they are particularly susceptible to experimental error in the region of the steep portions of the curves in Fig. 2. However, if the analytical values for the contents of lysine, arginine, and tyrosine (20) are accepted, it is clear that the maximum base-binding capacity of wool should be considerably greater than the highest values reported in this or any previous study.

SUMMARY

1. The amounts of hydrochloric acid and potassium hydroxide combined by wool, in solutions of two concentrations of formaldehyde, have been determined and compared with earlier determinations in the absence of formaldehyde. The effect of the presence of formaldehyde has been determined both in the absence and presence of salt.

2. The effects of formaldehyde on the dissociation curve of wool have been shown to be consistent, for the most part, with existing ideas as to the combination of this substance with the amino groups of lysine. There are indications that the guanidino groups of arginine also combine with formaldehyde, at least when salt is not present.

3. Because of the strong basicity of the guanidino groups, in neither the present study nor any earlier one has the maximum base-binding capacity of wool been measured.

mm per gm., the pK of the phenolic groups in wool lies near 10.5. This is only slightly higher than the value in the free amino acid.

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GASOMETRIC AND PHOTOMETRIC MEASUREMENT OF ARGINASE ACTIVITY

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Hunter and Dauphinee (1) outlined a titrimetric method by which arginase activity could be measured. This method utilized a principle employed by Edlbacher and Röthler (2) and involved a 30 minute reaction of arginase on a dilute solution of arginine (varying between 0.0238 and 0.0149 M) buffered with phosphate at pH 8.4, subsequent incubation with urease to hydrolyze the urea formed by the action of arginase, aeration of the resulting ammonia into acid, according to the technique of Van Slyke and Cullen (3), and titration of the residual acid. This method was later improved by Hunter and Downs (4) by use of a buffer mixture which was more efficient at pH 8.4, and employment of a constant, initial concentration of substrate (0.02225 M).

The present report outlines two procedures by which arginase activity can be measured. In both, the urea formed is determined as a measure of the arginine hydrolyzed. In the first, a gasometric procedure, Method I, the whole determination is conducted in the Van Slyke-Neill blood gas apparatus (5). Arginase acts on arginine for 10 minutes at pH 9.5 (near the optimum for arginase). The pH then is adjusted to 6.8 and urease is added to hydrolyze urea. The CO₂ liberated from urea is then measured as previously described by Van Slyke (6). The gasometric method is convenient when frequent, single arginase determinations are desired, as when one is following the effects of procedures on the purification or activity of the enzyme.

In the photometric procedure, Method II, the action of arginase takes place in test-tubes and the resulting urea is measured by Archibald's (7) procedure based on the color formed by the reaction of urea with α -nitrosopropiophenone. The photometric method is particularly convenient when numbers of simultaneous arginase determinations are done. Obviously the photometric method cannot be used to measure quantitatively the arginase content of preparations which contain urease.

In both methods the concentration of substrate is such that the amount of urea formed is proportional to the amount of enzyme present.

Van Slyke and Cullen (3) showed that the kinetic action of urease at a given pH was described by Equation 1

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$$(1) \quad E = \frac{1}{t} \left(\frac{1}{K_1} \log \frac{a}{a-x} + \frac{x}{K_2} \right)$$

where E represents the concentration of enzyme, t the time of reaction, a the initial concentration of substrate, and x the decrease in substrate concentration at time t . K_1 is the velocity constant of the combination of enzyme and substrate, K_2 the velocity constant of the decomposition

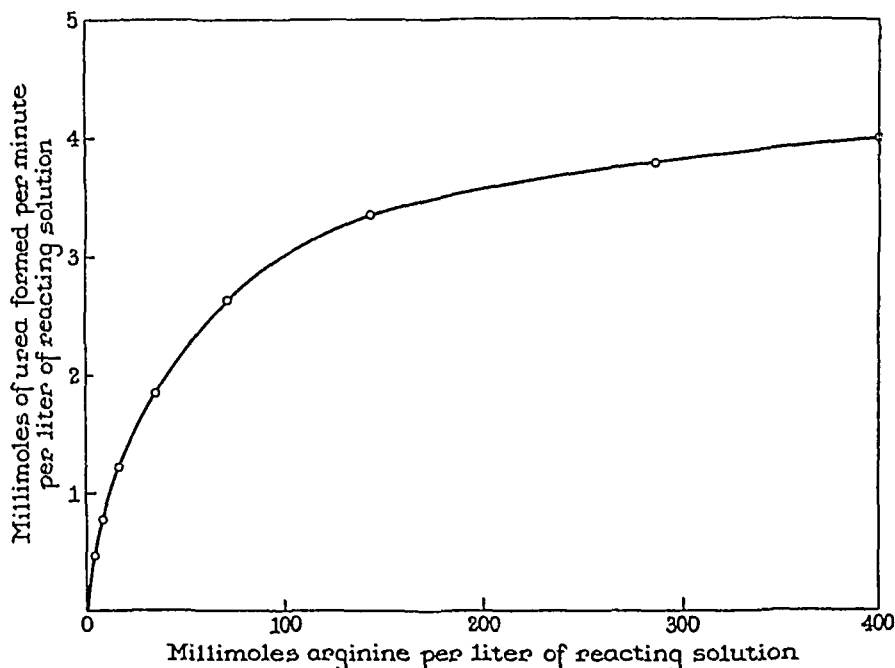


FIG. 1. Effect of concentration of substrate in the reaction mixture on the rate of hydrolysis of arginine at pH 9.5 and 25°. A 1 cc. portion of a 1:50 dilution of dog liver extract activated with manganous ion was mixed with each of 0.5 cc. portions of arginine, which was 3 times the concentration indicated by the figures on the abscissa. The analyses were conducted gasometrically.

of the combined substrate. We have found (8, 9)¹ that the same formula applies in a general way to the kinetics of arginase. Enzymatic decomposition of arginine appears to involve two successive stages: (1) combination of substrate and enzyme, and (2) decomposition of the combined substrate. The greater the concentration of arginine the shorter the interval required for formation of the complex. When the arginine concentration is sufficiently high, the formation of enzyme-substrate complex occurs so rapidly that the time involved in this step is insignificant com-

¹ A more detailed report (Van Slyke, Archibald, and Rieben) will be submitted later.

pared with the time taken by the decomposition. Under these conditions, for a limited reaction time, a of Equation 1 is so large in proportion to x that $a/(a - x)$ does not rise much above unity; hence $\log a/(a - x)$ does not rise much above zero, and the term $1/K_1 \log a/(a - x)$ may remain

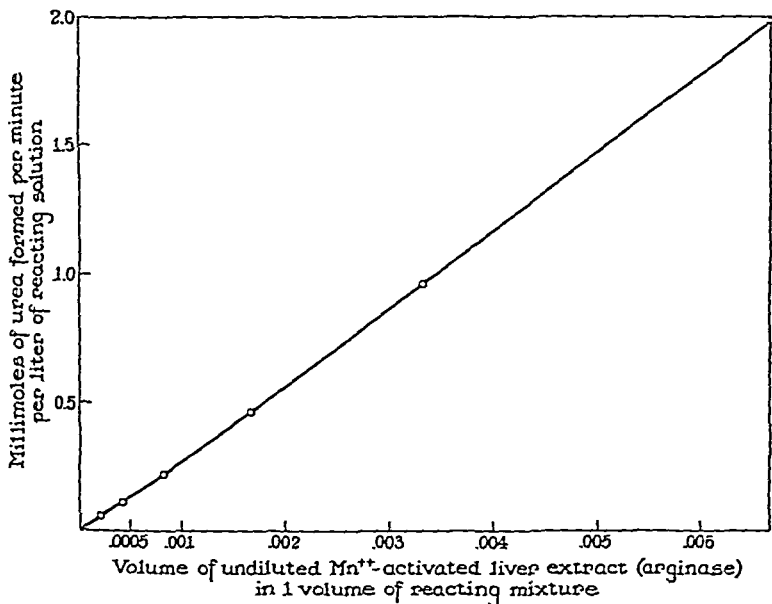


FIG. 2. Curve showing the proportionality between the amount of arginine hydrolyzed and the amount of enzyme present. Arginine concentration in the reacting mixture = 285 mM per liter. Reactions were conducted as outlined in the gasometric procedure.

insignificant compared with the term x/K_2 . The equation then simplifies to

$$(2) \quad E = \frac{x}{K_2 t}$$

As shown in Figs. 1 and 2, the conditions that validate Equation 2 are met for practical purposes when the concentration of the arginine substrate (a) is 0.28 M and the pH is 9.5 the amounts of urea formed (x) in the short reaction period ($t = 5$ minutes) used are then directly proportional to the amount of arginase (E). The measurement of enzyme activity under these conditions has the advantages of rapidity in operation and simplicity in calculation.

The action of arginase on arginine is strongly inhibited by the presence

of ornithine (8-16), one of the products (17). However, if the concentration of arginine is large compared with that of ornithine (as is the case in the following procedures when only a small fraction of the arginine is decomposed), the retardation resulting from formation of ornithine is insignificant.

Selection of Arginase Unit

Edlbacher and Röthler (2) define as a unit of arginase that amount which in 1 hour at 38° will decompose 10^{-5} mole of arginine (*i.e.* will liberate urea equivalent to 0.34 mg. of ammonia) when the arginase is added to a mixture of 5 cc. of buffer of pH 9.5 containing glycine, sodium hydroxide, sodium chloride, and 10 cc. of 1 per cent arginine carbonate.

This definition has the following disadvantages.

1. Because the volume of enzyme solution added is not specified, the concentration of substrate in the final mixture is not defined.

2. Because a low concentration of substrate is employed, the decomposition of substrate will not be proportional to the amount of enzyme present. Hence a calibration curve must be employed.

3. The time of action of the enzyme is long; hence considerable inactivation of arginase may take place during the determination.

Hunter and Dauphinee selected (1) a larger unit which they defined as that amount of arginase which in $\frac{1}{2}$ hour at 37° will liberate 0.5 mg. of urea N from 10 mg. of arginine N, at pH 8.4 (buffered with 0.5 M phosphate and with an initial arginine concentration varying between 0.0149 and 0.0238 M and in a volume varying from 7.5 to 12 cc.). This unit shares with that of Edlbacher and Röthler the first two and, to a smaller extent, also the third of the disadvantages above. Hunter and Downs (4) redefined the unit of Hunter and Dauphinee as one-tenth that amount of arginase which in $\frac{1}{2}$ hour at 37° will liberate urea equivalent to 2.5 mg. of N. However, Hunter and Downs specify a total volume and initial substrate concentration of 8 cc. and 0.02225 M respectively, and state that the unit is identical in magnitude with that defined by Hunter and Dauphinee.

Other units of arginase activity have been defined ((18); (19), see p. 510; (20-22)).

There appears to be some advantage in being able to calculate the arginase activity directly from the amount of a product of the reaction without having to construct a calibration curve. For this reason we have deliberately chosen a new *unit of arginase activity* which we define as *that amount which in 1 minute at 25° and pH 9.5 (by glass electrode) and with a substrate concentration of 0.285 M will decompose 1 micromole (10^{-6} mole) of arginine (*i.e.*, will liberate 1 micromole of urea (0.06 mg.) containing 0.028 mg. of nitrogen).*

Although hepatic arginase might be very slightly more active at pH 9.7 than at pH 9.5, the activity falls off much more rapidly at values slightly higher than the optimum than at those slightly lower than optimum. Furthermore, most devices now available for measuring pH are less reliable at pH values higher than 9.5 than at lower ones. Therefore, pH 9.5 has been selected for activity measurements. This is believed to be as near to the maximum as is safe to recommend for a routine method.

I. GASOMETRIC METHOD

Apparatus

Van Slyke-Neill blood gas apparatus (5). The mercury should be free of traces of other metals such as copper, so as to minimize the amount of mercury which can pass into solution.

Two bulbs of 50 or 100 cc. capacity, each with a stop-cock at one end, as described by Austin *et al.* (23, 24), for anaerobic handling of fluids over mercury.

Two stop-cock burettes, 10 cc.

One stop-cock pipette, 1 cc.

Reagents

Arginine solution, 0.85 M, pH 9.5 (by glass electrode). To 9.00 gm. of arginine monohydrochloride add 1.6 cc. of 18 N NaOH (CO_2 -free) and make up to 50 cc. with water. The solution is stored in a closed bulb over Hg to prevent a fall in pH due to absorption of CO_2 . Before use, it is transferred to a stop-cock burette fitted with a soda lime tube.

H_2SO_4 , 1.2 N.

1.6 M KNaHPO_4 , 21.8 gm. of KH_2PO_4 + 10.5 cc. of 18.0 N (CO_2 -free) NaOH to 100 cc. with water. This solution is stored over Hg. Before use it is transferred to a stop-cock burette protected above with a tube of soda lime and fitted at its tip with a rubber ring.

Urease, 10 per cent solution of Squibb's double strength urease in 50 per cent glycerol.²

Caprylic alcohol.

Brom-thymol blue, 0.4 per cent aqueous solution.

5 N NaOH.

² The presence of canavanine (25) and arginase in the preparations of jack bean urease will not interfere with the determination of urea, because the pH at which the urease is allowed to act is lower than that at which the canavanine (or arginine) is hydrolyzed by arginase. Hence, the dialyzed urease (25) need not be employed. The preparation should contain at least 0.15 Sumner unit per mg. The activity of a given preparation can be determined readily in 15 minutes without special apparatus by the colorimetric method of Van Slyke and Archibald (23).

Procedure

Dilution of Arginase Preparation—The dilution of enzyme should be such that its activity is approximately equal to that of a 1:25 dilution of neutralized fresh beef liver extract prepared according to the directions of Hunter and Dauphinee (1); i.e., it should contain about 2 to 6 arginase units per cc. If such an extract is activated with manganese, dilutions will need to be 1:50 to 1:100. Arginase rapidly loses activity on dilution³ with water unless an excess of activating metal ion is present; therefore, if no activating metal has been added, dilution should take place *immediately* prior to the activity measurement (within 2 minutes of the beginning of the arginase action).⁴ Dilutions should be made in 0.85 per cent saline to minimize the precipitation of proteins. Any protein which comes out of solution carries arginase activity with it. Consequently aliquot portions of a dilution possess measurably different activities unless the protein is evenly distributed among the aliquots.

Reaction of Arginase and Arginine—0.5 cc. of the arginine solution (pH 9.5) is introduced into the chamber of the blood gas apparatus from a stop-cock burette fitted at its lower end with a rubber ring. The cup of the chamber is then partly filled with mercury. With the aid of a stop-cock pipette ((27), p. 125, Fig. 3), also fitted at its lower end with a rubber ring,⁵ 1 cc. of the diluted solution of arginase is introduced slowly enough so that it will form a layer over the arginine solution. The stop-cock of the chamber is sealed with mercury. The mercury in the chamber is lowered *at once*, raised, then lowered again to the 50 cc. mark. During the lowering the solutions of enzyme and its substrate become mixed and the action of the arginase starts. At the moment the lowering of the mercury begins a stop-watch is started. After 2.5 minutes the temperature in the water jacket is recorded and exactly 0.5 cc. of 1.2 N H_2SO_4 is measured into the chamber cup, followed by 1 drop each of brom-thymol blue and caprylic alcohol. After exactly 5 minutes enzyme action the cock above the reaction chamber is opened sufficiently to allow the acid (but no air) to enter the chamber and mix with the contents. This lowers the pH of the mix-

³ D. D. Van Slyke and R. M. Archibald, unpublished results to be presented later.

⁴ In some cases it is preferable to dilute the enzyme preparations only one-tenth as much, and to use one-tenth the volume of enzyme solution (delivered from a rubber-tipped capillary 0.1 cc. pipette). This is especially true when one is dealing with (a) unactivated preparations of arginase which rapidly lose activity when highly diluted, or (b) dilutions prepared from glycine extracts of liver which contain proteins not readily soluble in water or dilute saline. In this case, prior to the measurement of p_i , 1.5 instead of 0.5 cc. of 1.3 N H_2SO_4 are added.

⁵ Pipettes employed for measuring these diluted enzyme solutions must be cleaned frequently to avoid inclusion of air bubbles.

ture to 2.3 and thereby stops the enzyme reaction, irreversibly inactivates the enzyme, and sets free slight amounts of CO_2 unavoidably present in the reagents.

Removal of Preformed CO_2 from Reaction Mixture—After admission of the acid, the upper cock is sealed with mercury, and the evacuated chamber is shaken 2 minutes with the mercury meniscus near the 50 cc. mark to extract CO_2 from the solution. Mercury is then admitted from the leveling bulb until it fills the lower quarter of the chamber. Then, without stopping the inflow of mercury, the upper cock is opened, admitting air into the chamber. The admission of mercury into the chamber is continued until all the gases have been completely driven out through the upper cock, but is stopped before any of the solution enters the cup. The air is admitted in order to dilute the CO_2 gas in the chamber and prevent its reabsorption by the solution. If the upper cock were kept closed until the extracted CO_2 was compressed at the top of the chamber, some of the extracted CO_2 would go back into solution. The above procedure of extraction and ejection removes about 96 per cent of the CO_2 from the solution. It is repeated once, making the removal complete in so far as measurable amounts of CO_2 are concerned.

Hydrolysis by Urease of Urea Formed by Arginase—After expelling all but a small bubble of air, 0.5 cc. of KNaHPO_4 solution is introduced from a stop-cock burette. The stop-cock of the chamber is closed and sealed with mercury, and the contents of the chamber are mixed by once lowering and raising the mercury in the chamber. The pH should be 6.8, as indicated by the slight green tinge of the indicator. The cup is washed with 1.2 N H_2SO_4 , then with water, and 0.5 cc. of 10 per cent urease solution is introduced into the chamber and mixed with its contents by lowering and raising the mercury in the chamber. After 5 minutes 0.5 cc. of 1.2 N sulfuric acid is added, the mercury is lowered to the 50 cc. mark, and the CO_2 extracted and measured according to the directions of Van Slyke and Neill ((24), p. 277-278). p_1 is read with the gas at 2 cc. volume. The lower cock of the chamber is opened, and 0.3 cc. of 5 N NaOH is admitted from the cup to absorb the CO_2 . The aqueous meniscus is lowered below the 2 cc. mark, and 1 minute draining time is allowed; the meniscus is then brought back to the 2 cc. mark and pressure p_2 is read (see the procedure for CO_2 in plasma (24), p. 284-285).

Determination of c Correction by Blank Analysis—A blank determination is run in which the arginase is added after the 0.5 cc. of 1.2 N H_2SO_4 has been added and mixed. The 5 minute incubation period of arginase is omitted, but in all other regards the procedure is as outlined above. This blank corrects for the small amount of urea which may be present in the

preparation of arginase, as well as for CO_2 in the phosphate and urease, and any small degree of hydrolysis of arginine by jack bean arginase present in the preparation of urease employed. The $p_1 - p_2$ of the blank is the c correction.

Each determination requires about 15 minutes.

Between analyses the cup and chamber are rinsed out, first with 1.2 N H_2SO_4 and then with water. It is important to make sure that all urease is removed from the chamber walls or inactivated before proceeding with the next analysis.

If enough CO_2 is formed to give a pressure reading above 350 mm., the ammonium carbonate resulting from hydrolysis of urea will have raised the pH sufficiently above 6.8 to permit some action of jack bean arginase on the large amount of unhydrolyzed arginine present during the period when only urease should be acting. Hence if P_{CO_2} is greater than 250 mm., the procedure should be repeated with a more dilute arginase solution.

Calculation for Gasometric Procedure

The CO_2 pressure, P_{CO_2} , exerted at 2 cc. volume by the CO_2 liberated from urea formed from arginine and then hydrolyzed by urease is calculated as

$$(3) \quad P_{\text{CO}_2} = p_1 - p_2 - c$$

where p_1 and p_2 are the manometer readings obtained in the analysis and c is the value of $p_1 - p_2$ obtained in the blank analysis.

Units of arginase in 1 cc. of diluted arginase solution expressed as " E (dilute)" are calculated by the equation

$$(4) \quad E (\text{dilute}) = P_{\text{CO}_2} \times \frac{A}{T}$$

where T is the incubation time in minutes and A is the factor in Column 2 of Table I. The arginase concentration, " E (original)," in the original undiluted arginase preparation is calculated as

$$(5) \quad E (\text{original}) = E (\text{dilute}) \times V$$

where V is the volume to which 1 volume of the original arginase preparation was diluted. A is the product of $B \times C$, the values for which appear in Columns 3 and 4 respectively of Table I. The factors under B in Column 3 are those by which the values of P_{CO_2} in mm. are multiplied to give micromoles (moles $\times 10^{-6}$) of arginine decomposed (= micromoles of urea formed or of CO_2 measured). These factors are given by Van Slyke and Sendroy ((28), Table X for $S = 3.5$ cc., $a = 2.0$ cc.). C (Column 4, Table I) cor-

rects for the effect of temperature on the activity of the arginase during the incubation period.

TABLE I

Factors by Which Mm. of PCO_2 Are Multiplied to Give Micromoles of Arginine Decomposed by Sample at 25°

S = 3.5 cc. a = 2.0 cc.

Temperature (1)	$B \times C = A$ (2)	B (3)	C (4)
°C.			
10	0.3165	0.1266	2.50
11	0.2918	58	32
12	712	50	17
13	523	43	03
14	373	36	1.92
15	237	29	82
16	102	22	72
17	0.1968	15	62
18	836	08	52
19	707	02	42
20	591	0.1196	33
21	487	90	25
22	384	83	17
23	295	77	10
24	228	71	05
25	165	65	1.00
26	102	60	0.95
27	038	54	90
28	0.0977	49	85
29	926	43	81
30	876	38	77
31	827	33	73
32	778	28	69
33	741	23	66
34	710	18	635
35	678	12	610
36	647	07	585
37	622	02	565
38	598	0.1098	545
39	570	93	522

It is to be noted that "*E (dilute)*" of Equations 3 and 4 is 1.5 times the enzyme concentration, *E* (Equations 1 and 2), in the reacting mixture of arginase and arginine.

II. PHOTOMETRIC METHOD

Apparatus

Dialysis units described by Hamilton and Archibald (29).

Photometer.

Reagents

Arginine solution, pH 9.5, prepared and stored as indicated above for the gasometric method.

Metaphosphoric acid, 15 per cent solution.

α -Isonitrosopropiophenone,⁶ 3 per cent solution in 95 per cent ethyl alcohol.

Sulfuric-phosphoric acid mixture, 90 cc. of concentrated sulfuric acid, c.p., and 270 cc. of syrupy phosphoric acid (reagent grade) are poured into 600 cc. of water in a Pyrex container. The volume is then adjusted to 1000 cc.

Stock standard solution of urea (4 mM), 24.0 mg. of urea in 100 cc. of water. Store in the ice box. Prepare fresh monthly.

Working standard urea solution (0.4 mM). Dilute 1 volume of the stock urea solution to 10 volumes with phosphoric-sulfuric acid mixture on the day the working standard is to be used. The working standard contains 0.4 micromole of urea per cc.

Procedure A for Photometric Method

This procedure is used when the arginase solution to be tested contains so much protein that the latter would interfere with the photometric urea determination. In this procedure the urea formed is separated from the proteins by rapid dialysis, or by precipitation with metaphosphoric acid. Procedure A is used, for example, in estimating arginase activity of erythrocytes.

Dilution of Arginase Preparation—Immediately before the activity measurement, the arginase solution to be tested is so diluted that the resultant solution contains 0.1 to 0.4 arginase unit per cc. (This activity is about that of a 300-fold dilution of Hunter and Dauphinee's beef liver extract, or of a 600-fold dilution of this extract activated with manganese.)

Reaction of Arginine and Arginase—The solutions of arginine and diluted arginase are brought to measured room temperature. To 0.5 cc. portions of the arginine solution, adjusted to pH 9.5, in 5 cc. test-tubes are added 1 cc. portions of freshly diluted preparation of each arginase solution to be tested.⁵ The substrate is mixed thoroughly with the enzyme immediately after the latter has been added. Exactly 10 minutes after the

⁶ Obtained from Anachemia, Ltd., Montreal, Canada, and 70 East 45th Street, New York 17, New York.

arginine and arginase are mixed 1 cc. of sulfuric-phosphoric acid mixture is added and mixed. This stops the action of arginase.

A control for the reagent blank is prepared by adding the acid to the arginine before, instead of after, the addition of the arginase.

Separation of Generated Urea from Colloids of Digest. (a) *Separation by Dialysis*—A 2 cc. portion of each acidified digest and control is placed in the inner portion of a dialysis unit (29), a 15 cc. portion of the sulfuric-phosphoric acid mixture is placed in the outer compartment, and the unit is assembled and dialysis is continued with rocking for 3 hours (the total volume of solution in dialysis is 17 cc.). The dialysate is "dialysate a."

(b) *Alternative Removal of Colloids by Precipitation with Metaphosphoric Acid*—In this case the action of the enzyme is stopped by the addition of 1 cc. of 15 per cent metaphosphoric acid. After 15 minutes standing the mixture is centrifuged and the supernatant is decanted into a clean tube. To a 2 cc. portion of the supernatant add 15 cc. of the sulfuric-phosphoric acid solution; mix and filter to obtain "filtrate b."

Photometric Measurement of Urea—A 10 cc. portion of each dialysate a or filtrate b, including the controls, is pipetted into a 20 cc. test-tube.

Standards are prepared by adding to 20 cc. test-tubes 1, 3, 5, 7, and 9 cc. of the working standard and 8, 6, 4, 2, and 0 cc. respectively of the phosphoric-sulfuric acid mixture. To each standard is then added a 1 cc. portion of acidic arginine solution prepared by diluting 1.15 cc. of the 0.85 M arginine (pH 9.5) to 5 cc. with the phosphoric-sulfuric acid mixture.⁷ The standards contain 0.4, 1.2, 2.0, 2.8, and 3.6 micromoles of urea per 10 cc.

To each tube containing an aliquot of dialysate, filtrate, or a standard, 0.5 cc. of the 3 per cent solution of α -isonitrosopropiophenone is added. After the contents are mixed, the tubes are stoppered and heated 1 hour in a boiling water bath, in the manner outlined by Archibald (7) for the colorimetric determination of urea. The tubes are cooled in the dark, and thereafter protected from light. The optical density is read at a wavelength of 540 m μ against the reagent blank set at zero.

The 10 cc. aliquot of the control, treated as above, serves as reagent blank.

Calculation for Photometric Method, Procedure A

From the standards a curve is prepared of optical densities against micromoles of urea present in 10 cc. of standard solution. From the observed

⁷ Usually the dilute arginase solution adds nothing to the reagent blank. When this is the case, control solutions need not be prepared; the reagent blank is then prepared by measuring 9 cc. of the phosphoric-sulfuric acid into a 20 cc. test-tube and adding 1 cc. of the acidic arginine solution. Arginine decreases appreciably the color developed in the reaction between urea and α -isonitrosopropiophenone. Hence, approximately the same amount of arginine is added to the blank and standards that is present in the 10 cc. aliquots of dialysate a or of filtrate b.

optical density of the unknown, U , the number of micromoles of urea in the analyzed 10 cc. aliquot is read on the curve. The arginase concentration, E , in units per cc. of the diluted arginase solution, is calculated from U as

$$E \text{ (dilute)} = 0.212UC$$

For the original, undiluted arginase preparation the activity in units per cc. is calculated as

$$E \text{ (original)} = 0.212UCV$$

C (from Table I) and V have the same significance as in the calculation for the gasometric method

$$\text{Factor } 0.212 = \frac{2.5}{2.0} \times \frac{17}{10} \times \frac{1}{10}$$

Of the constituent factors, 2.5/2.0 is the ratio of total acidified digest volume to the volume of aliquot used; 17/10 is the ratio of total fluid in the dialyzed or precipitated mixture to the aliquot used for urea measurement; 1/10 is the ratio of the standard time unit (1 minute) to the time used for the digestion.

Procedure B for Photometric Method

When the concentration of protein in the diluted arginase solution is less than 0.2 mg. per cc. (as is the case when arginase preparations from dog or beef liver are tested), the protein need not be removed. In this case the enzyme reactions are conducted in 25 cc. test-tubes and are stopped by the addition of a 20 cc. portion of the phosphoric-sulfuric acid mixture to each 1.5 cc. portion of digest. 10 cc. aliquots of the mixture are treated with color reagent as indicated above.

The calculations are:

$$E \text{ (diluted)} = 0.215UC$$

$$E \text{ (original)} = 0.215UCV$$

$$\text{Factor } 0.215 = \frac{21.5}{10} \times \frac{1}{10}$$

The 21.5/10 is the volume ratio of total acidified digest to the aliquot used for urea measurement; 1/10 and U , C , and V are the same as in Procedure A.

Because of the very small amount of enzyme required for the activity measurement, and because the presence of small amounts of protein other than enzyme does not interfere, this photometric method is well adapted to the study of the distribution of arginase among the various separable fractions of cells. Obviously the procedure can be adapted readily to

measurement of smaller amounts of arginase by decreasing (5- to 20-fold) the volumes of all reagents and by increasing (2- to 6-fold) the reaction period. However, if the reaction of a diluted arginase solution is prolonged for 30 or more minutes, appreciable inactivation may occur and somewhat diminish the estimated activity, unless nickel, cobaltous, or manganous ion is added.

Discussion of Methods

As can be seen from an inspection of Fig. 1, the concentration of arginine which enables activated liver arginase of dog to act at its maximum velocity at pH 9.5 is higher than the 0.285 M concentration used. However, when arginine in 0.285 M concentration is employed as substrate, the fraction of arginine decomposed during the reaction is not great enough to depress significantly the rate of urea formation, which remains proportional to the amount of enzyme present. If the concentration of substrate were increased, say 4-fold, the amount of acid and alkali required to adjust the pH for the later parts of the procedure would be undesirably large. Furthermore, the cost of the arginine required for the determination would be increased.³

At the substrate concentration employed, the pH optimum for hepatic arginase is between 9.5 and 9.9. Because arginine is itself an excellent buffer at this pH, there is no need to add either phosphate or glycine to the mixture in which arginase acts.

In view of the variable effect of (a) the source (31) and of (b) the pH of activation³ on the pH optimum of arginase, one would not expect preparations of arginase from several sources, or arginase from one source prepared by different methods, to give activities by Hunter's method (which measures activity at pH 8.4) that would be exactly proportional to activities indicated by our method (with measurement at pH 9.5). For example, jack bean arginase, which, when activated with manganese, has a pH optimum near 8.4 would, as compared with liver arginase, give relatively higher results by Hunter's method than by ours. Nevertheless, the convenience and simplicity of the method outlined above have made it appear desirable to employ these conditions for activity measurements and to re-define the unit in terms of these conditions.

Because of the rapid loss of activity in dilute solutions of unactivated preparations,³ use of the short (5 to 10 minutes) incubation periods permits a more accurate estimation of the activity of unactivated preparations than can be obtained by using the much longer incubation periods (30 to 60 minutes) previously employed by many workers. If, for example,

* A large portion of the arginine in the reaction mixtures can be recovered and purified with flavianate (30) and then used again for other activity determinations.

activity during the first 5 minutes of incubation were represented by 100, that in the second 5 minutes by 80, 64 for the third, 51 for the fourth, 41 for the fifth, and 33 for the sixth, the apparent activity for a 30 minute period would be only the average of these, *viz.* 61.5.

Discussion of Gasometric Method

Stock, Perkins, and Hellerman (31) have shown that jack bean arginase, either alone or after addition of activating ions such as Mn^{++} , Ni^{++} , or Co^{++} , has no activity at pH lower than 6.8.

Hellerman and Stock (32) have shown that liver arginase, either alone or after treatment with Mn^{++} ion, has almost no activity at pH 6.0 and very little at pH 6.8. For urease, in contrast, the optimum pH lies near 6.8, when urea concentration is 0.15 M (the optimum pH is higher when urea is more dilute (33)). Hence, urea (even in the presence of large amounts of arginase) can be determined at pH 6.8 with jack bean urease without interference from jack bean arginase. Residual traces of liver arginase will not interfere appreciably unless Ni^{++} or Co^{++} ions are present in amounts sufficient to activate the liver arginase (32). Richards ((32), p. 774) employed pH 6.0 for the determination of urea by urease in the presence of arginine. We have used pH 6.8, because the time required for urease to decompose the last traces of urea present (33) is much longer at pH 6.0 than at 6.8. Thus 5 minutes action of urease at pH 6.8 suffices, instead of the 2 hours at pH 6.0 employed by Hellerman and Stock. At pH higher than 6.8 there is danger of action of jack bean arginase. Even crystalline urease, unless repeatedly recrystallized, prepared from jack bean is reported to contain arginase (31).

Kossel and Dakin (17) defined arginase as an enzyme which hydrolyzed arginine to urea and ornithine. Some authors (34) have measured arginine before and after action of their enzyme preparations and have assumed that the disappearance of arginine was a measure of arginase. However, enzymes other than arginase are capable of destroying arginine. For example, Hills (35) demonstrated that some Gram-positive cocci were capable of splitting arginine to ornithine, CO_2 and NH_3 , and that urease was not present in sufficient amounts to explain the production of the NH_3 and CO_2 . He named the responsible enzyme arginine dihydrolase. Niven *et al.* (36) appear to have worked with a similar system and reported that most *Streptococci* (Lancefield Group A to G whether hemolytic or not), except *viridans*, liberate NH_3 and CO_2 from arginine but do not attack urea or creatine. Arginine decarboxylase of *Bacillus coli* (37, 38) and *B. cadaveris* (39) in the presence of *Streptococcus faecalis* acting on arginine is reported to yield agmatine and CO_2 (37). Horn (40) reports that "arginine desimidase" of *B. pyocyaneus* splits arginine to give a 4.9 per

cent yield of citrulline. Although some bacteria have been reported to contain arginase (34, 41, 42), proof of the presence of this enzyme must rest not alone on the ability of the enzyme preparation to remove arginine from solution nor on its ability to produce NH_3 or CO_2 from arginine, but rather on production of urea. When CO_2 or NH_3 formed by action of urease is used to measure the urea, it is therefore desirable that the enzymes of the arginase preparation be inactivated and any CO_2 or NH_3 formed be removed, before urease is added to determine the urea.

No enzyme other than arginase is known to yield urea from arginine.⁹

In the gasometric method described above, any CO_2 liberated from arginine by enzymes other than arginase, during the initial incubation at

TABLE II
Comparison of Arginase Activities of Dog Liver and Human Erythrocytes by Gasometric and Photometric Methods

Source of arginase	Method	Arginase, units per cc. glycerol extract after activation with manganous ion	
Dog liver	Gasometric	340	
	Photometric		
	Procedure A, dialysis	336	
	" " pptn.	345	
	" B, protein not removed	350	
Human erythrocytes	Photometric	Non-activated	Activated
		units per cc. cells	units per cc. cells
J. W.	Procedure A, dialysis	0.09	9.1
"	" " pptn.	0.09	9.3
R. A.	" " "	0.18	6.7
D. R.	" " "	0.03	3.9

pH 9.5, as well as the CO_2 inevitably present in the reagent solutions, is expelled prior to the estimation of the urea formed from arginine by action of arginase. Hence only arginase (and not arginine dihydrolase or arginine decarboxylase) is measured by this method. Likewise when the colorimetric method for urea is applied, the activity of arginase alone is measured.

Neither the gasometric method, as described in the preceding pages, nor

⁹ Krebs and Eggleston (43) have reported that *Corynebacterium ureafaciens* splits urea from arginine, citrulline, agmatine, creatine, creatinine, allantoin, allantoic acid, hypoxanthine, and uric acid. It has not been demonstrated whether the enzyme, which in this case splits arginine, is indeed arginase, or whether it is the same enzyme which forms urea from the other guanidine derivatives.

the photometric method is suited to the determination of arginase in preparations which contain urease, since the urea formed would be partially destroyed before it could be determined.

The gasometric method can, however, be modified to measure arginase in the presence of urease, by omitting the extraction and expulsion of CO_2 just prior to the addition of the phosphate buffer. When this alternative is employed, the initial extraction of CO_2 is omitted also in the determination of the blank. This modification results in higher blanks and has the disadvantage that results for arginase will appear too high if there are present enzymes, other than arginase, which liberate CO_2 from arginine.

Results

Table II compares the values for the activity of a preparation of dog liver arginase activated with Mn^{++} ion obtained by the gasometric method, and by the photometric method both with and without dialysis or precipitation of protein. The liver arginase was activated fully by heating to 58° for 20 minutes with 0.05 volume of a 20 per cent solution of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (9). Values are given for the arginase in erythrocytes of several adult human males before and after activation with manganous ion. The activation of erythrocytes took place in 2 hours at room temperature (23°). A 1:10 to 1:20 dilution of activated cells is used for the photometric determination of activity.

SUMMARY

Gasometric and photometric procedures are described for measurement of arginase activity by the rate of urea formation.

The gasometric procedure is carried out entirely in the Van Slyke-Neill manometric apparatus at room temperature. The arginase acts for 5 minutes at pH 9.5; then the pH is dropped to 6.8, at which arginase does not act, and the urea formed is measured by the CO_2 set free by urease.

In the photometric procedure the reaction conditions for arginase are similar, but the urea generated is measured by the photometric method of Archibald (reaction with α -isonitrosopropiophenone).

In both procedures the pH, arginine concentration, and reaction period are such that the rate of urea formation is directly proportional to arginase concentration.

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THE ASSAY OF ANIMAL TISSUES FOR RESPIRATORY ENZYMES

V. THE MALIC DEHYDROGENASE SYSTEM*

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The assay of animal tissues for the components of the malic dehydrogenase system can be carried out by applying the principles previously outlined in other applications of the homogenate technique (1-4). A very high degree of cell disruption is accomplished by means of homogenization in hypotonic medium (2, 5). The homogenate is used at high dilution (1), and the desired phase of metabolism is studied by the addition of particular substrates and cofactors at concentrations which make the chosen reaction limiting (1, 2).

In the case of the malic dehydrogenase system, the problem is complicated by the fact that two factors hamper the operation of the system. The first is the accumulation of oxalacetate. The equilibrium between malate and oxalacetate is strongly in favor of malate, so that, unless the oxalacetate is removed, the oxidation of malate is rapidly inhibited. If the oxalacetate were oxidatively removed, the measurement of malate oxidation would include an unknown quantity of oxalacetate oxidation. Fortunately Banga and Szent-Gyorgyi discovered that the addition of glutamate to the reaction mixture "fixed" the oxalacetate so that malate oxidation could proceed ((8), see also (9)). The second complicating factor is the fact that the malate system requires DPN (diphosphopyridine nucleotide, coenzyme I, cozymase) and this compound is rapidly destroyed in homogenates (10). Here the problem was solved by Mann and Quastel (11), who found that nicotinamide, a constituent of DPN, markedly inhibits the breakdown of the coenzyme and thus facilitates the study of the DPN-linked dehydrogenases. Cytochrome *c* has to be restored to homogenates to overcome the lowering of its concentration by simple dilution (2, 3). Similarly, DPN has to be added to homogenates in order to compensate for its dissociation from its specific proteins, although the nature of this dissociation may need to be reexamined in view of recent findings for other cofactors (12, 13).

So far as we are aware, the malic dehydrogenase system has not been

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¹ As Cohen has pointed out (6), their finding constituted evidence for what was later recognized as transamination (7).

previously studied with all of the above considerations in mind. Straub (9) did not make use of nicotinamide, while Mann and Quastel (11) did not employ glutamate or cytochrome *c*. In the present study, the optimum concentration of all of the reactants was sought and the test system was based upon the use of homogenates in order that a technique for the assay of various normal and pathological tissues could be worked out.

By adding malic dehydrogenase, the limiting factor becomes DPN-cytochrome *c* reductase² instead of malic dehydrogenase, and both enzymes can be conveniently assayed on one small sample of tissue.³

EXPERIMENTAL

All of the work reported in this paper was based upon the measurement of the rate of oxygen uptake by means of the conventional Warburg apparatus at 38°. Considerable work was carried out with flasks with side arms, so that the oxidation of malic acid could be initiated after equilibration by adding the coenzyme (DPN) from the side arm. In this work, the reactants were at room temperature before being placed in the bath. More recently we have used flasks with no side arms, and have placed them in a pan of cracked ice just before adding the homogenate. The cold flasks are then transferred to the 38° bath and the measurements are begun after a 6 minute equilibration. When the rate of uptake is low, the first 5 minute reading may be in error but subsequent readings represent the attained rate. It can be stated that either method gives valid results for this particular system, owing to the stability of the malic dehydrogenase, as will be brought out later. We prefer the flasks with no side arms, and most of the data in this paper were obtained with the technique in which ice is employed to control the reaction prior to the beginning of measurements.

In the preceding publication (17) we adopted a nomenclature which recognized the differences between homogenates prepared in hypotonic and in isotonic media originally pointed out by Elliott and Libet (5). All of the present work was done with "water homogenates" so as to obtain maximum disruption of cells, except in one series of experiments which demonstrates the basis for this procedure.

Most of the work was done on rat liver, and, in most cases, the animals were used at an age of between 2 and 4 months. Tissues from such animals appear to yield the highest values.

Cytochrome *c* was prepared in the laboratory and standardized spectro-

² This name is modified from the previous term employed (14) in keeping with the present tendency to use the term DPN.

³ The assay conditions were stated in a laboratory manual in 1945 (15) but no data were given. The conditions have been slightly modified since their application by Anderson *et al.* (16).

photometrically (15). The malic acid was obtained from the Pfanstiehl Chemical Company, while the glutamic acid and nicotinamide were Merck products.⁴ The chief problem in this work has been the supply of coenzyme (DPN), since each experiment requires so much of the material. Our early work was made possible by generous gifts of DPN from Merck, while more recently Dr. G. A. LePage of this laboratory, using a procedure based on that of Williamson and Green (18), has kindly made adequate supplies

TABLE I

Optimum Conditions for Malic Dehydrogenase System

The source of enzyme was rat liver; 0.2 ml. of a 5 per cent water homogenate was added to each flask. The data for each compound added were obtained from a separate experiment with a freshly prepared homogenate. Thus the variation between animals is the source of the variation in the plateau values obtained with the various compounds. Each compound was studied at varying concentrations with all other compounds held constant and at the optimum value. The concentration of each component selected for the assay technique is indicated by bold-faced type, and if two concentrations are thus indicated, the chosen concentration is between the values given. The QO_2 was calculated on the basis of the best 10 minute period.

Malate		Glutamate		Nicotinamide		Phosphate		DPN†		Cytochrome c	
Molarity	QO_2	Molarity	QO_2	Molarity	QO_2^*	Molarity	QO_2		QO_2	Molarity $\times 10^{-3}$	QO_2
								<i>γ per ml.</i>			
0	8.8	0	17.2	0	57.6	0	100.4	0	0	0	14.3
0.0083	69.4	0.0083	53.3	0.0033	74.0	0.010	102.4	33	28.6	0.33	36.1
0.0167	77.4	0.0167	60.8	0.0067	75.8	0.020	94.2	100	62.4	0.67	47.9
0.033	82.8	0.033	71.6	0.0134	79.4	0.027	96.6	333	94.2	1.33	66.3
0.058	82.8	0.058	69.8	0.02	79.0	0.033	92.0	1000	112.0	2.66	85.1
0.083	71.4	0.083	72.6	0.033	79.6	0.050	84.4	2000	112.0	4.00	91.8
										5.33	92.2

* QO_2 based on the second 10 minute period to accentuate the effect of protecting diphosphopyridine nucleotide (DPN) by addition of nicotinamide.

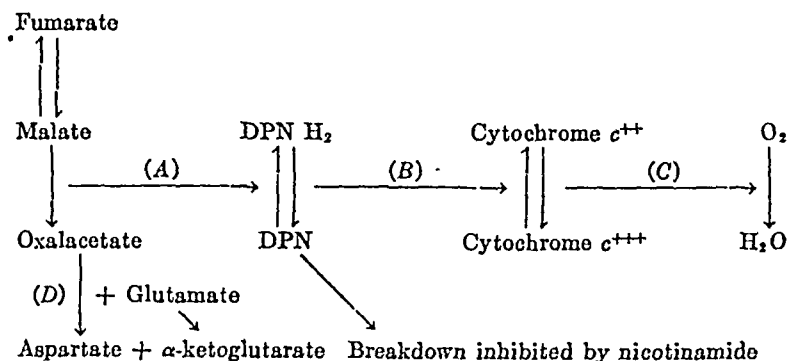
† Preparation from Merck and Company, Inc., about 40 per cent DPN.

available. We are also indebted to Dr. F. Schlenk for a primary DPN standard. Recently the Schwarz Laboratories, Inc., has placed a 40 per cent DPN product on the market, and we find it satisfactory. The non-metallic impurities in DPN preparations having 40 to 80 per cent DPN do not appear to interfere with the reaction system here employed, and it is merely necessary to determine the plateau level of the DPN available.

⁴ We are glad to acknowledge the cooperation of Merck and Company, Inc., in supplying us with certain compounds for this work. The supplies of DPN were especially helpful, and were obtained from Merck through the courtesy of Dr. D. F. Green.

If metals are present, the plateau level will be depressed. Neutral solutions of 1 per cent DPN have been kept refrigerated for 1 week without apparent loss and all other solutions employed appear to keep indefinitely when refrigerated. However, those that will support molds, etc., are made up in 50 cc. quantities and are not used after 2 weeks.

Components of System—According to present information, the malic dehydrogenase system consists of malic dehydrogenase, DPN, DPN-cytochrome *c* reductase,² cytochrome *c*, and cytochrome oxidase. Although further dissection of the system may be anticipated (19), it is fairly safe to assume that there is no dissociation of components beyond those listed, when fresh homogenates are used, and the data of this paper support such an assumption. Earlier investigators had shown the importance of additions of cytochrome *c* (9), DPN (20), glutamate (8), and nicotinamide (11), as well as the reasons for adding these substances. It remained to adapt these findings to the study of the reaction in tissue homogenates and to find the optimum concentration of each component. This has been done in a large series of experiments and the data are condensed in Table I. The system does not appear to need additional sources of inorganic phosphate, but an appreciable amount is used in order to increase the buffering capacity of the medium somewhat. The data clearly demonstrate the need for each of the other components, and the effect of each can be explained on the basis of the accompanying diagram in which reactions A, B, C, and D are



catalyzed by enzymes which are furnished by the homogenate, and are considered to represent malic dehydrogenase, DPN-cytochrome *c* reductase, cytochrome oxidase, and transaminase respectively. Enzymes in the homogenate determine the rate of oxygen uptake when the components listed in Table I are all added in excess. Proof that this is true is given in Table II, in which the rate is shown to be proportional to the amount of homogenate added. If one of the enzymes is definitely the limiting component, the data in Table II show that the system is probably valid for

assay work. Table III shows that fumarate is almost as good as malate, so that fumarase is probably present in amounts nearly as great or possibly greater than the other components. However, malic acid is the more desirable substrate. Previous assays for cytochrome oxidase (2) show that this enzyme is present in larger amounts than required here, and is probably not limiting. The rate falls off considerably even in the present system, and

TABLE II

Relation of Oxygen Uptake to Tissue Concentration

The source of enzyme was mouse liver; a 2.5 per cent water homogenate was prepared. The components of the assay system were added on the basis of the data in Table I, the sodium salts of all acidic compounds, adjusted to pH 7.4 to 7.6, being used. The actual amounts were as follows: 0.8 ml. of 0.1 M phosphate, 0.3 ml. of 0.5 M glutamate, 0.3 ml. of 0.5 M malate, 0.3 ml. of 0.1 M nicotinamide, 0.3 ml. of 1 per cent solution of diphosphopyridine nucleotide (DPN) preparation (40 per cent DPN), 0.3 ml. of 4×10^{-4} M cytochrome c, plus 0.1 to 0.6 ml. of 2.5 per cent homogenate and water to make a final volume of 3.0 ml. The center cup contained 0.2 ml. of 2 N NaOH. Temperature, 37°. Flasks with no side arms were used, chilled just before adding the homogenate and equilibrated 6 minutes at 37°.

Fresh liver per flask, mg.	2.5	5.0	7.5	10.0	12.5	15.0
O ₂ per best 10 min., microliters	11.4*	25.0	36.7	48.8	56.8	65.6
QO ₂	91.2	100.0	98.0	97.6	91.0	87.5

* The second 10 minute period was chosen; in all other cases the value for the first 10 minutes was the best.

TABLE III

Comparison of Substrates for Malic Dehydrogenase Assay

Conditions as in Table II, except that 0.2 ml. of 5 per cent mouse liver homogenate was used in all flasks, and the source of malate was varied.

Substrate	Q _{O₂}	
	0.2 ml. 0.5 M substrate	0.3 ml. 0.5 M substrate
Malate (Sargent)	62.6	63.8
l-Malate (Pfanstiehl)	96.2	95.0
Fumarate (Eimer and Amend)..	88.0	89.6

loss of DPN must be in part responsible. Application of the test system to brain yielded much lower results than for liver, and this might be attributed to the DPNase of brain (4, 11), or it might be thought to be related to a deficiency of transaminase. One test of such possibilities is afforded by summation experiments, such as shown in Table IV, in which the ability of each tissue to oxidize malic acid is measured separately and in combination with the other tissue. The results are in close agreement, suggesting

that the same component is limiting for both tissues. Thus the assay of each tissue is probably valid. Since the data are based upon the first readings, before much DPN is lost or much oxalacetate has accumulated, it is considered virtually certain that the assay is a determination of either malic dehydrogenase or DPN-cytochrome *c* reductase; that is, of *A* or *B* in the diagram. Evidence presented below indicates that *B* is in excess, so that the assay is probably a measure of malic dehydrogenase in most instances.

Further Resolution of Components—In order to determine with greater certainty the limiting component of the malic dehydrogenase system, it is necessary to study the effect of adding malic dehydrogenase to the system. If malic dehydrogenase is the limiting component, an increased rate will be observed. Pure malic dehydrogenase would be the proper reagent here, but it is convenient to use a muscle extract of the type originally used for

TABLE IV
Summation of Malic Dehydrogenase Activity

5 per cent water homogenates of rat brain and rat liver were prepared, and the malic dehydrogenase activity of each tissue was determined separately and in the presence of the other tissue, 0.2 ml. of each homogenate being used. The conditions were as in Table II. Two different rats were used. Data are expressed as the micro-liters of oxygen taken up per 20 mg. of fresh tissue per 10 minutes. In rat liver this is also the Q_{O_2} (15).

Liver activity	Brain activity	Calculated sum	Observed sum
122.4	27.8	150.2	153.2
92.8	27.2	120.0	125.8

glycolysis studies. Such a preparation was used by Green, Needham, and Dewan (20) using rabbit muscle, and was referred to as a mutase. Their preparation was shown to catalyze the reaction



but to be unable to catalyze the reactions which lead to oxygen uptake (20). When such a preparation was added to the system described in Table II, an increase of about 50 per cent in the rate of oxygen uptake was observed. Thus it seems likely that in the system in Table II the limiting component was malic dehydrogenase, and that the system represents an assay for this enzyme. As might be expected, assay of pigeon breast muscle showed that the concentration of malic dehydrogenase was high and this has been used for large scale preparation of crude malic dehydrogenase as follows: Breast muscle from eleven pigeons was extracted with 4 volumes of distilled water in a Waring blender in the cold. The mince was allowed to stand 24 hours in the cold and was then filtered by gravity in

the cold room during the next 24 hours. The clear red filtrate was treated at -5° with 2 volumes of cold acetone and the precipitate was centrifuged down, placed in dialysis tubing, and dialyzed through four changes of distilled water. The insoluble protein was filtered off and the clear solution was placed in a series of 13×100 mm. tubes, frozen, and stored in the frozen state at about -20° . The activity of the resulting preparation is shown in Table V. When the malic dehydrogenase is present in excess, the limiting reaction is almost certainly reaction *B*, catalyzed by DPN-cytochrome *c* reductase, since the attained rate is still well below the cytochrome oxidase rate. Thus it is possible to assay for both *A* and *B* with little additional effort. The data in Table V include assays on primary hepatomas, in comparison with normal liver. Aside from the fact that the hepatomas

TABLE V
Assay for DPN-Cytochrome c Reductase

Water homogenates of rat liver and hepatoma were tested at a level of 10 mg. of fresh liver or 20 mg. of hepatoma per flask, the conditions for the malic system approximately as described in Table II being used. In addition, varying amounts of crude malic dehydrogenase preparations were added in order to test the capacity of the homogenate to catalyze the reaction between reduced DPN (diphosphopyridine nucleotide) and oxidized cytochrome *c*. The data are expressed as microliters of oxygen taken up per 20 mg. of fresh tissue per 10 minutes.

Malic dehydrogenase preparation added	Liver	Hepatoma*
None.....	109.6	9.7, 13.4
Pigeon breast muscle extract, 0.1 ml...	163.2	12.8, 17.4
" " " " 0.3 " ..	173.0	
" " " " 0.5 " ..	180.8	

* Two different specimens.

were very low in comparison with normal liver, the data are interesting technically, because the hepatomas exhibit such a narrow margin between the two enzymes that one may ask whether there is sufficient cytochrome reductase in the hepatoma to yield a valid malic dehydrogenase assay. However, summation experiments such as were done with brain in Table IV gave very slight increases. It would be desirable to add pure crystalline malic dehydrogenase instead of the crude material, but this material has not as yet been obtained. The crude material which has been used thus far is incapable of independently catalyzing oxygen uptake in the reaction mixture and is virtually free of cytochrome reductase and cytochrome oxidase but probably contains transaminase. The presence of the latter is desirable.

Properties of System—It is desirable to know something about the properties of the system in order to know what precautions are needed to avoid

loss of activity and in order to coordinate the system with other systems; e.g., coupled phosphorylation (21, 22). For the latter, we have used water homogenates, and the data herein show that water homogenates can be used for the present system. However, a comparison with the isotonic homogenate is of interest and is given in Table VI, which includes the effect of DPN and cytochrome *c* on both types of homogenate. It is rather surprising to find that the isotonic homogenate is nearly as dependent upon added DPN and cytochrome *c* as the water homogenate, since in the case of the succinic system the isotonic homogenate was relatively inde-

TABLE VI

Malic Dehydrogenase Activity in Water Homogenates and in Isotonic Homogenates

Contiguous portions of tissue from the livers of individual rats were homogenized in distilled water or in isotonic NaCl at a concentration of 5 per cent, and the malic dehydrogenase activity was measured by the test system described in Table II, except that saline was added to the flasks containing water homogenates in an amount equal to that contained in the isotonic homogenates.

Preparation	Variations in reaction mixture		
	Complete system	Cytochrome omitted	Diphosphopyridine nucleotide omitted
	microliters O ₂	microliters O ₂	microliters O ₂
Water homogenate.....	120.0	8.8	0
Isotonic homogenate.....	93.0	13.2	9.2
Water homogenate, median lobe...	129.2		
“ “ left lobe.....	124.0		
Isotonic homogenate, median lobe.	102.6		
“ “ left lobe.....	109.2		

pendent of added cytochrome (17). Table VI includes data comparing the results of assays on adjacent portions of liver by each technique, as a control on the possible variation in samples. The agreement was excellent in each case. The water homogenate is considered to be the better technique for preparing tissue for assay (*cf.* (4)).

In the previous work with coupled oxidation and phosphorylation the system was extremely unstable, and brief incubations at 38° in the absence of the reaction mixture resulted in inactivation (21). Unpublished data on the octanoate system (17) suggest even greater instability. In these systems, speed and cold are necessary precautions in handling the homogenate. The malic system is in marked contrast, as shown in Table VII. No loss in malic dehydrogenase occurs in a 5 per cent water homogenate in 15 minutes at 38° or in 24 hours at 0°. This may well be a result of the fact that in the malic system we know the identity of the coenzyme and replace

TABLE VII

Stability of Malic Dehydrogenase System in Liver Homogenates

Water homogenates containing 5 per cent of fresh rat liver were tested for malic dehydrogenase activity in the test system described in Table II. Aliquots were incubated for various periods of time at 0° and at 38° and then tested under the standard conditions. The incubation time and temperature refer to the treatment of the homogenate before its addition to the test system.

Experiment No.	Incubation time and temperature	Crude cozymase added	Oxygen uptake in test system
3-1*	Control	0	0
	"	3000	95.8
	"	1000	81.0
	5 min., 38°	1000	72.4
	10 " 38°	1000	75.6
	15 " 38°	1000	77.0
	24 hrs., 0°	1000	88.2
3-26†	Control	0	0
	"	1000	95.0
	"	3000	120.0
	"	6000	119.2
	24 hrs., 0°	3000	116.2

* Cozymase from Merck and Company, Inc.

† Cozymase from Dr. LePage, prepared according to Williamson and Green (18).

TABLE VIII

Effect of Certain Inhibitors on Malic Dehydrogenase System

Water homogenates containing 5 per cent of fresh rat liver were tested for malic dehydrogenase activity in the test system described in Table II. The inhibitors were added to the reaction mixture after all other reactants except the homogenate had been added. Each experiment was carried out with a liver from a different rat. The values represent Q_{O_2} .

Experiment No.	Control	Inhibitor, final concentration			
		Fluoride, $M/75$	Malonate, 0.01 M	Dinitrophenol, 0.0001 M	Dinitrophenol, 0.01 M
3-27	122.8	125	133.0		
3-29	119.0	123.0		111.0	102.0
3-30	90.8		74.6	90.8	83.6
4-1	106.0		107.8		

it in excess, while in the "labile" systems there may be as yet unidentified coenzymes.⁵ The data show that no special precautions are necessary in the case of the malic system.

⁵ We were unable to reactivate octanoste oxidation with DPN of proved activity in the malic system, in contrast to previous findings (23).

It was desirable to use fluoride in the phosphorylating system (21) and we wished to know whether this would inhibit the fully activated malic system. The data in Table VIII show that fluoride even in a high concentration does not inhibit malate oxidation. In studying the components of the Krebs cycle, it is desirable to add malonate to block the cycle at the succinate stage, if malonate does not interfere with the chosen reaction. We conclude from Table VIII that malonate does not inhibit malic dehydrogenase in the complete system (but see (24)). Dinitrophenol was found to inhibit oxidative phosphorylation in unpublished experiments, but comparable levels had a negligible effect on malate oxidation, as shown in Table VIII.

Another question of considerable interest is the action of the system upon other substrates. Oxalacetate and α -ketoglutarate are formed in the

TABLE IX
Oxidation of Lactate in Malic Dehydrogenase System

Each tissue was studied at a level of 10 mg. of fresh tissue prepared as a 5 per cent water homogenate, with conditions as in Table II, except that when lactate was the substrate, no glutamate was added. The data are expressed as microliters of oxygen taken up per 20 mg. of fresh tissue in 10 minutes.

Substrate	Tissue studied			
	Liver	Kidney	Brain	Hepatoma
Malate.....	119.0, 106.0,* 92.8, 90.8	89.6, 84.6	27.2, 27.8	17.4
Lactate.....	57.4, 51.8, 53.6, 35.0	34.0	6.6	5.6

* When glutamate was omitted, the value fell to 21.8 (compare Table I), which is considerably lower than the corresponding value of 51.8 for lactate.

reaction, although the amounts are very small in the first 10 or 15 minutes. However, the absence of Mg^{++} and adenosine triphosphate (ATP) from the reaction mixture makes any significant oxidation of these substances very improbable. Moreover we have been unable to increase the oxygen uptake on malate by additions of ATP or various ion combinations to the present "complete" system. On the other hand, lactate is oxidized effectively in this system by liver or kidney homogenates, but not by brain or hepatoma homogenates (Table IX). This does not detract from the malate assay, but may offer an opportunity to study lactate oxidation or, by use of lactate plus malate, to study the requirements of the Krebs condensation.

Assay Results—A number of assays have been carried out on rat liver, and the results together with data from some other tissues are presented in Table X. It is clear that hepatoma is low in comparison with normal

liver, and preliminary data on a number of other tumors show that they fall in the same range. It is interesting to find liver more active than kidney, since for a closely related enzyme, succinic dehydrogenase, the opposite was true (2). It is probably incorrect to assume that the change represented by the comparison of liver with hepatoma is characteristic of the cancer process, however, since for succinic dehydrogenase a similar change was noted, while the opposite change was noted in the case of skin (25). What is needed is the widest possible survey of normal and cancer tissues, and more important, the relation between the level of the respiratory

TABLE X
Malic Dehydrogenase Assays

The tissues were prepared for study in the form of 5 per cent water homogenates, and were added at a level of 10 mg. of tissue per flask, with conditions as in Table II. The data are expressed as microliters of oxygen taken up per 20 mg. of fresh tissue in 10 minutes.

Tissue	No of samples	Malic dehydrogenase, average and range
Rat liver	18	103.5 (73.2-125.4)
" hepatoma	5	15.1 (9.7-20.2)
" brain	2	27.5 (27.2-27.8)
" kidney	3	81.4 (70.0-89.6)
Human kidney*	3	19.3 (17.3-20.7)
Mouse liver ...	2	100.6 (97.6-103.6)
" brain ...	1	42.2
Pigeon breast muscle	1	107.0†

* Biopsy specimens showing mild arteriosclerosis, total sample 150 mg., of which 130 mg. were used for another purpose.

† A portion of this sample was stored as whole muscle for 2 weeks at 4° and assayed a second time, giving the figure 103.8 for malic dehydrogenase.

enzymes and the complementary enzymes which deplete the energy balance (22).

DISCUSSION

The measurements of malic dehydrogenase activity as outlined in this paper tend to yield a figure which is actually a measure of the concentration of the specific catalytic proteins involved. They are essentially a measure of the *potential* activity of the enzyme being considered. With the total electron transport occurring through physiological carriers, and expressed as the Q_{O_2} , one has a figure which can be discussed in terms of the earlier measurements of Q_{O_2} on slices or for other enzymes in homogenates. Assays for cytochrome oxidase showed (2) that this enzyme is present in

concentrations much higher than found here for cytochrome reductase or for malic dehydrogenase, while the data thus far indicate that cytochrome reductase is present in excess over malic dehydrogenase. Thus each assay depends upon the fact that the electron-transporting enzymes increase in concentration as the pathway approaches oxygen. The physiological significance for this is probably that there is a certain amount of convergence of systems as oxygen is approached. In the case of cytochrome oxidase, the plateau level of cytochrome *c* was very high, and in the present case, the saturating amount of DPN was very high. In each case the observation may be due to the fact that the added electron carrier has to shuttle between two reactants which are widely separated in space, one of which is particulate. So long as we are able to supply the missing diffusible components of the systems in sufficient quantity, we are able to compensate for the dilution of the cellular contents, and to permit the limiting enzyme to work at its maximum capacity. One must recognize the possibility, however, that the concentration of the "excess" enzyme may not actually be in wide enough excess to give the maximum values for the "limiting" reaction, although if enough of the mediating carrier is present, the permissible margin should be quite low.

If it is true that the data constitute a reliable measure of the potential malic dehydrogenase and cytochrome reductase activity of the samples, one must consider the physiological significance of these data. When it is considered that the Q_{O_2} of liver slices is probably not outside the range of 6 to 10, it is clear that under these conditions only a small fraction of the malic dehydrogenase (average Q_{O_2} , 103) is being used in the respiring tissue slice. Without questioning the significance of the data on slices at this point, and recognizing the importance of "control factors" in metabolism, we nevertheless attach significance to the individual enzyme assays on the theory that the total amount of any given enzyme in a tissue must be a mathematical function of the average actual use to which the enzyme is put in the given tissue. Such an interpretation implies that the concentration of a given enzyme in a tissue will be increased or decreased under certain conditions. Recent studies have demonstrated the alteration of enzyme concentration in yeasts, depending upon the presence of the specific substrate (26). That the concentration of certain respiratory enzymes may actually change in the course of training is suggested by Palladin (27) who has summarized data on this subject. Thus Chepinoga (28) reported that the succinic dehydrogenase of rabbit muscles was increased 50 to 100 per cent by training. Negative results in the case of studies on malic dehydrogenase by the same author (29) must be discounted in view of the lack of adequate assay techniques at that time. Recently we have shown marked increases in succinic dehydrogenase and cytochrome oxidase in rat liver

and brain of new-born rats (30) and Meyer, McShan, and Erway (31), using the same methods, found that the corpora lutea of rat ovaries fluctuate between an average succinate Q_0 value of 18.5 during diestrus to 46.8 in pregnancy. These authors state "there is a direct correlation between the succinic dehydrogenase activity and the high and low levels of lutein tissue function." These fluctuations in succinic dehydrogenase activity in highly specialized tissue with periodic function constitute strong support for the assumption that the measurements of *potential* enzyme activity are physiologically significant.

For a complete understanding of the significance of the concentration of a respiratory enzyme, however, its activity must be considered in relation to the activity of the enzymes which make use of the energy of oxidation. This is particularly important in interpreting the findings in cancer tissue, and will be discussed in a separate paper when more cancer data are available. The present system can now be coordinated with the total system for the study of oxidative phosphorylation (21, 22).

SUMMARY

1. The homogenate technique has been extended to the assay for malic dehydrogenase and DPN-cytochrome *c* reductase.
2. The proposed technique is based upon the measurement of the rate of oxygen uptake by use of the conventional Warburg apparatus and water homogenates of animal tissues at a level of 2 to 20 mg. of fresh tissue per flask.
3. The complete chain of reactants includes malic acid, malic dehydrogenase, diphosphopyridine nucleotide (DPN, coenzyme I), DPN-cytochrome *c* reductase, cytochrome *c*, cytochrome oxidase, and oxygen. Purified preparations of DPN and cytochrome *c* are added in excess.
4. Side reactions are controlled to some extent by adding glutamic acid to decrease oxalacetate accumulation and by adding nicotinamide to decrease DPN breakdown.
5. Muscle extracts containing malic dehydrogenase but lacking cytochrome enzymes were added to the complete system to shift the assay from malic dehydrogenase to cytochrome reductase.
6. Some of the properties of the system were studied, and assays for liver, hepatoma, and a few samples of other tissues were reported.

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HYDROLYSIS PROCEDURES FOR THE DETERMINATION OF TRYPTOPHANE IN PROTEINS AND FOODSTUFFS BY THE MICROBIOLOGICAL PROCEDURE*

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A survey of the literature shows that a number of different methods have been used for the estimation of tryptophane in foodstuffs. Greene and Black (1), Wooley and Sebrell (2), and Stokes *et al.* (3) have recently described microbiological methods for the determination of this amino acid. Difficulties are encountered in the microbiological method and in many other methods of assay because tryptophane is subject to destruction during liberation from the sample.

We wish to report in this paper studies on the reliability of different hydrolysis procedures. *Lactobacillus arabinosus* was used as the organism.

EXPERIMENTAL

The basal medium was the same as that used by Schweigert *et al.* (4) for leucine and valine assays, except that casein hydrolysate and cystine (50 mg. and 2 mg. respectively, per tube) were used in place of the purified amino acids. Pure *l*(-)-tryptophane was used for the standard curves at the following levels: 0, 2.5, 5, 7.5, 10, 12.5, 15, and 20 γ per tube. The curves obtained are similar to those previously reported for tryptophane. The blank titration was 0.5 ml., while the 20 γ level of tryptophane produced acid equivalent to 14.0 ml. of 0.1 N sodium hydroxide after an incubation period of 2 days. Two samples of *dl*-tryptophane were found to possess one-half the activity of samples of *l*(-)-tryptophane; these results confirm the finding of Wooley and Sebrell (2). A sample of *d*(+)-tryptophane showed no activity in water solution.

When samples were treated with acid or alkali, the following conditions were used. 1 to 5 mg. of tryptophane, 100 to 200 mg. of purified protein, or 1 gm. of meat sample was autoclaved at 15 pounds pressure with 25 ml. of 2 N acid or 10 ml. of 1 or 5 N sodium hydroxide. After the solutions were autoclaved they were cooled and diluted to 100 ml. 10 to 25 ml.

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aliquots were neutralized and diluted to 100 ml. and duplicate aliquots containing 1, 2, and 3 ml. were used for assay. To several of the samples, quantities of pure tryptophane were added before hydrolysis. The percentage of the added tryptophane recovered (as estimated by comparison with samples without this addition) is recorded in Table I under the heading "Recovery."

No measurable destruction or racemization of pure *l*(-)-tryptophane or of *d*(+)-tryptophane occurred when they were autoclaved with distilled water for 2 hours.

Treatment of *l*-tryptophane for 2 hours with 2 *N* acid resulted in a loss of about 25 per cent of its activity. Since similar losses occurred with the racemic form and *d*-tryptophane gave no activity after treatment with acid, most of the loss of activity must be due to destruction. This destruction is not a result of humin formation, since no carbohydrate was present. Values for tryptophane in casein after acid hydrolysis for 2 hours were reproducible but the results were extremely erratic in the case of meat samples. Consequently, the recoveries showed similar variability. The effect of the duration of acid hydrolysis on the apparent tryptophane content of a sample of pork is recorded in Table I. The values increased at first and then decreased as the period of autoclaving was prolonged. Similar results were obtained with lower concentrations of sulfuric acid and with hydrochloric acid on several other meat samples.

Decreased activity of both *l*- and *dl*-tryptophane likewise occurred after autoclaving with alkali. This loss of activity, which is of the same order as that produced by acid, is partially due to racemization, since alkaline treatment of *d*-tryptophane produced racemization. When the duration of alkali treatment was increased to 20 hours there was no greater loss than was observed after 2 hours.

The results obtained after casein was hydrolyzed for 1 hour with 5 *N* sodium hydroxide were relatively reproducible and largely independent of duration of treatment. Lower values were obtained when 1 *N* sodium hydroxide was used. The values for casein, obtained by alkaline hydrolysis, are approximately half those obtained by acid hydrolysis. This difference has been explained on the basis of racemization (1).

The reproducibility of the tryptophane values obtained when meat samples were hydrolyzed with alkali was not as good as with casein but was considerably better than with acid treatment. Values did not decrease upon further digestion up to 8 hours. The tryptophane values for the alkaline hydrolysates of meat were about half those obtained with acid.

Since treatment with either acid or alkali decreased the activity of pure tryptophane and since the values obtained with meat samples were erratic, enzymatic methods were used for the liberation of tryptophane. Greene

and Black (1) obtained low values when samples were hydrolyzed for 24 hours with pancreatin. Wooley and Sebrell (2) used pepsin followed by trypsin and erepsin. Pancreatin was used in some of the present studies, while in others it was combined with preparations from hog or rat mucosa.

TABLE I
Effect of Acid and Alkali in Liberation of Tryptophane

Sample	Hydrolytic agent	Duration of auto-claving	No. of samples	L-Tryptophane	Recovery
		<i>hrs.</i>		<i>per cent</i>	<i>per cent</i>
l(-)-Tryptophane.....	H ₂ O	2	6	95 -103	
d(+)-Tryptophane.....	"	2	2	0	
l(-)-Tryptophane.....	2 N HCl	2	6	62 -82	
"	2 " H ₂ SO ₄	2	2	76	
"	5 " NaOH	2	10	62 -84	
"	5 " "	20	3	67 -70	
dl-Tryptophane	2 " HCl	2	3	32 -38	
"	5 " NaOH	2	4	31 -38	
"	5 " "	20	2	35 -43	
d(+)-Tryptophane.....	2 " HCl	16	2	0	
"	5 " NaOH	16	5	12 -16	
Casein.....	2 " HCl	0.5	1	0.54	
"	2 " "	2	4	0.91-0.98	
"	1 " NaOH	3	2	0.37(Both)	
"	5 " "	1	1	0.54	
"	5 " "	2	5	0.41-0.45	
"	5 " "	5	9	0.45-0.53	70
"	5 " "	8	2	0.50-0.51	78
"	5 " "	20	1	0.56	67
Pork 3.....	2 " HCl	2	11	0.09-0.16	80-142
" 3.....	2 " H ₂ SO ₄	5	2	0.06	88
" 3.....	2 " "	2	2	0.07	102
" 3.....	2 " "	5	2	0.04	76
" 3.....	2 " "	10	2	0.01	20
" 3.....	5 " NaOH	3	3	0.04-0.07	98-126
" 3.....	5 " "	5	1	0.06	
Veal 1.....	2 " HCl	5	2	0.09-0.13	
" 1.....	5 " NaOH	5	3	0.06-0.09	72- 98
" 1.....	5 " "	8	1	0.06-0.07	69

The pancreatin was Merck, U. S. P. grade, the hog intestinal mucosa a commercial product furnished by Wilson and Company. Rat intestinal mucosa was fresh scrapings from the inner surface of the small intestine of adult rats. The duration of treatment, the amounts of the enzymes added, and the effects of shaking were studied on several purified proteins

and meat samples. For each 1 gm. sample of animal tissue or 100 to 200 mg. of purified protein the amount of enzyme indicated in Table II was used. The samples were placed in 100 ml. volumetric flasks, diluted with water to approximately 50 ml., and the pH adjusted initially to 8.2 with 0.1 N sodium hydroxide. The samples were then covered with toluene and placed in an incubator at 37° for varying periods of time. After the samples were hydrolyzed they were cooled to room temperature, diluted to 100 ml., and 10 to 25 ml. aliquots were taken. They were neutralized, diluted to 100 ml., and duplicate aliquots of 1, 2, and 3 ml. were used for assay. Corrections were made for the tryptophane added by the enzymes.

The activity of *l*-tryptophane is not impaired when it is incubated for periods up to 5 days with pancreatin alone or when combined with mucosal preparation. With casein in the amount mentioned above it was found that when 2 days of incubation were used the tryptophane activity increased with increasing amounts of pancreatin up to 25 mg. When the pancreatin level was 25 mg. the amount of tryptophane liberated was increased during periods of incubation from 1 to 5 days. Further incubation was without effect; the tryptophane values remained the same after 8 days as they were after 5 days of incubation. The addition of hog mucosa to the digestion mixture did not increase the tryptophane values above those produced by pancreatin alone. However, shaking of the digestion mixture with the two enzyme preparations present produced "maximal" liberation in 1 day.

Fibrin and wheat gluten appear to be completely hydrolyzed by pancreatin alone in 1 day. However, this was not true with lactalbumin, since definite increase in tryptophane liberation from this protein was noted after the addition of hog mucosa and shaking.

Digestion of samples of pork with pancreatin alone, even for periods as long as 5 days, gave wide variations in the amount of tryptophane liberated. Such variation is also reflected in the irregular recoveries. The addition of hog or rat mucosa even in high amount and for prolonged periods of time did not eliminate this variability. Without shaking, the amount of tryptophane liberated increased up to 5 days, but even with 5 days incubation the results were scarcely better in the runs containing mucosa than in the experiments employing pancreatin alone. If the samples were shaken continuously, the liberation achieved in 1 day was as great as that in 5 days without shaking, and the variability between samples was practically eliminated.

Continuous shaking of digests containing only pancreatin did not constitute an entirely satisfactory procedure, as can be seen from the figures for Pork 1. In these experiments, even with continuous shaking, the tryptophane was not completely liberated in 1 day, values on the 4th and

TABLE II
Enzymatic Liberation of Tryptophane under Various Conditions

Sample	Method of treatment			Shaker*	Time	No. of samples	L-Tryptophane	Recovery
	Pan-creatin	Hog mucosa	Rat mucosa					
	mg.	mg.	mg.		days		per cent	per cent
Casein.....	4			—	2	1	1.00	
"	8			—	2	5	1.09-1.16	
"	25			—	1	2	1.17-1.19	
"	25			—	2	1	1.22	
"	25			—	3	2	1.21-1.26	98
"	25			—	5	4	1.22-1.30	
"	25			—	8	2	1.28-1.30	
"	50	25		+	1	3	1.27-1.33	
Fibrin.....	50			—	1	2	3.18-3.24	
"	50	25		+	1	3	3.07-3.36	
"	50	25		+	2	3	3.09-3.29	
Wheat gluten.....	50			—	1	2	0.73-0.75	
" "	50	25		+	1	2	0.74-0.79	
" "	50	25		+	2	2	0.76-0.78	
Lactalbumin.....	100			—	3	2	2.13-2.33	
"	50	25		+	1	6	2.62-2.73	
					hrs.			
Pork 1.....	50			+	7	4	0.12-0.14	97-109
					days			
" 1.....	50			+	1	2	0.15-0.16	96-103
" 1.....	50			+	4	2	0.19	99-101
" 1.....	50			+	6	2	0.20	102-110
					hrs.			
" 1.....	50	25		+	7	4	0.16-0.18	106-119
					days			
" 1.....	50	25		+	1	3	0.19-0.21	95-103
" 1.....	50	25		+	4	4	0.20	98-109
" 1.....	50	25		—	5	6	0.14-0.19	
" 2.....	50	25		+	1	4	0.15-0.16	
" 2.....	50	25		+	2	4	0.15-0.17	
" 3.....	25			—	5	12	0.10-0.20	46-160
" 3.....	25		125	—	1	3	0.10-0.16	78-130
" 3.....	25		125	—	2	3	0.12-0.15	102-118
" 3.....	25		125	—	3	4	0.14-0.17	134
" 3.....	25		125	—	5	5	0.14-0.19	
" 3.....	25		125	—	8	1	0.17	
" 3.....	25	50		—	1	1	0.16	
" 3.....	25	50		—	2	1	0.16	
" 3.....	25	50		—	3	2	0.14-0.16	
" 3.....	25	50		—	5	5	0.10-0.19	
" 3.....	50	25		+	1	4	0.17-0.18	

* Samples digested in a shaker are designated by +, while samples which did not receive continuous shaking are indicated by —.

6th days being markedly higher. The addition of hog mucosa, with shaking, brings about complete liberation in 1 day but not in 7 hours. With an identical digestion mixture, 5 days without shaking yielded less complete hydrolysis than 1 day with shaking (average tryptophane values 0.17 and 0.20 per cent respectively). The results for pork sample (No. 2) also illustrate the completeness of hydrolysis with 1 day of shaking in the pres-

TABLE III
Tryptophane Content of Natural Foodstuffs

Material	No. of samples	Moisture	Fat	Protein	Tryptophane	Tryptophane in protein*	Values from literature
		per cent	per cent	per cent	per cent	per cent	per cent
Lactalbumin.....	9			95.9	2.66	2.77 (2.88)	2.3 (5), 1.79 (6, 2), 2.06(1)
Casein.....	3			85.2	1.31	1.54 (1.58)	1.8 (5), 1.20 (1), 1.07† (3), 1.20 (6, 2)
Fibrin.....	6			89.9	3.20	3.56	3.4 (5)
Wheat gluten.....	4			82.8	0.77	0.93	1.0 (5)
Soy bean oil meal..	3			43.9	0.76	1.73	1.6 (5)
Veal 1.....	4	74.0	5.3	19.2	0.21	1.10	1.39 (7)
" 2.....	3	74.5	3.0	21.7	0.24	1.11	
Pork 1.....	4	50.2	34.0	14.9	0.20	1.34	1.37 (1)
" 2.....	4	51.8	33.8	13.0	0.16	1.23	
" 3.....	3			15.0	0.19	1.23	
Beef Kidney 1....	3	79.2	3.0	14.5	0.19	1.34	1.81 (7)
" " 2....	3			14.6	0.21	1.44	
" tongue.....	3	66.6	16.9	16.0	0.19	1.19	
" liver.....	3	69.5	4.1	20.0	0.32	1.60	1.38 (3), 1.81 (7), 1.5 (5)
" Heart 1.....	3			18.1	0.24	1.33	1.41 (7)
" " 2.....	3			18.2	0.23	1.26	

* All values are calculated to 16 per cent N. The values in parentheses are calculated to 15.6 per cent nitrogen for casein and 15.4 per cent nitrogen for lactalbumin.

† Uncorrected.

ence of pancreatin and hog mucosa. There was no change in values when the period was extended to 2 days.

In Table III are presented tryptophane values for a few natural foodstuffs. These were obtained by digesting the material with 50 mg. of pancreatin and 25 mg. of hog mucosa. The samples were shaken for 1 day in a 37° incubator and diluted for assay as described above.

DISCUSSION

The problem of perfecting a microbiological assay for tryptophane depends largely upon the development of a satisfactory method for hydroly-

sis of the materials to be analyzed. The reliability and specificity of the tryptophane assay with *Lactobacillus arabinosus* have not been questioned by those working in this field. Although Wooley and Sebrell (2) have shown that indole can replace tryptophane, their work indicates that none of the materials used in this study would supply indole. When judging the adequacy of a hydrolysis procedure, it appears logical to select the procedure which produces the highest tryptophane value.

On this basis it is clearly evident that both acid and alkaline hydrolysis yielded values which were low for all the substances tested. Reproducible values were obtained for casein after acid hydrolysis but the known destruction of pure samples of tryptophane and of tryptophane in the meat samples after acid treatment renders the data for casein questionable.

The values obtained after hydrolysis with sodium hydroxide were less variable but they were low even if complete racemization is assumed. That racemization is a factor is evident from the activity of solutions of *d*-tryptophane after alkaline hydrolysis. There is good reason to believe that there is destruction of tryptophane after treatment with alkali because pure samples of *dl*-tryptophane, so treated, showed markedly less than the 50 per cent of the expected activity. The loss must be due to destruction, since these samples are completely racemized and gave the expected 50 per cent activity before hydrolysis. Wooley and Sebrell (2) and Horn and Jones (6) have not given serious consideration to destruction by sodium hydroxide. In the light of our results it seems possible to explain many of the differences between the two types of hydrolysis procedures which they used and between the chemical and microbiological determinations not only on the basis of incomplete hydrolysis or racemization but on varying degrees of destruction.

With alkaline hydrolysis the destruction does not increase with prolonged time. It is probably for this reason that most workers have assumed that only racemization was concerned and that, if alkaline autoclaving were continued long enough, values representing half the tryptophane present would result. This seems to be the reasoning behind the recommendation of Stokes *et al.* (3) to autoclave for at least 10 hours. They believe sodium hydroxide digestion to be satisfactory but the values they obtained were lower than those obtained by enzyme treatment. Greene and Black (1) employed 5 mg. of pancreatin (for each 0.1 gm. of dry protein) for 24 hours, and found lower values than those they obtained by autoclaving with barium hydroxide. This is not surprising in the light of the present work, which indicates that in the absence of shaking complete hydrolysis is not obtained by digestion with pancreatin alone even after periods of several days, except with a few materials.

Wooley and Sebrell (2) investigated pepsin, trypsin, and crepsin as hydrolytic agents. A digestion period of 4 days was employed. Their

results for casein and lactalbumin are lower than those reported in the present work. In general in order to obtain complete liberation of tryptophane in reasonable lengths of time it is necessary to combine mucosal preparations with pancreatin and shake the reaction mixture continuously. Our experience indicates that low values are associated with marked variation between aliquots of a single material, and suggests that hydrolysis has proceeded at varying rates due to uncontrolled factors such as particle size. Thus, it is possible that certain samples may require more than 1 day for digestion, but the samples tested in the present study have given maximal liberation with 1 day under the conditions outlined above.

The actual values obtained for several different samples are listed in Table III. Direct comparison can be made with the results obtained by other workers. The values for the meat samples are lower than those obtained by Beach *et al.* (7) by a chemical method. The values for casein and lactalbumin are higher than those obtained by Wooley and Sebrell (2) and Horn and Jones (6) by enzymatic digestion, and are generally higher than those reported by Greene and Black (1) by either of their methods. The values for casein and beef liver reported by Stokes *et al.* (3) by alkaline hydrolysis are lower than those reported by any of the investigators. Variations in results among workers are believed to be due to incomplete liberation in many of the samples tested or to loss of tryptophane through the method of hydrolysis.

SUMMARY

Liberation of tryptophane from proteins and foodstuffs has been effected by shaking the samples for 24 hours in a mixture of pancreatin and hog mucosa.

The tryptophane content of several proteins and foodstuffs was measured by *Lactobacillus arabinosus* after liberation by this method.

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A SYNTHESIS OF *l*(+)-GLUTAMINE*

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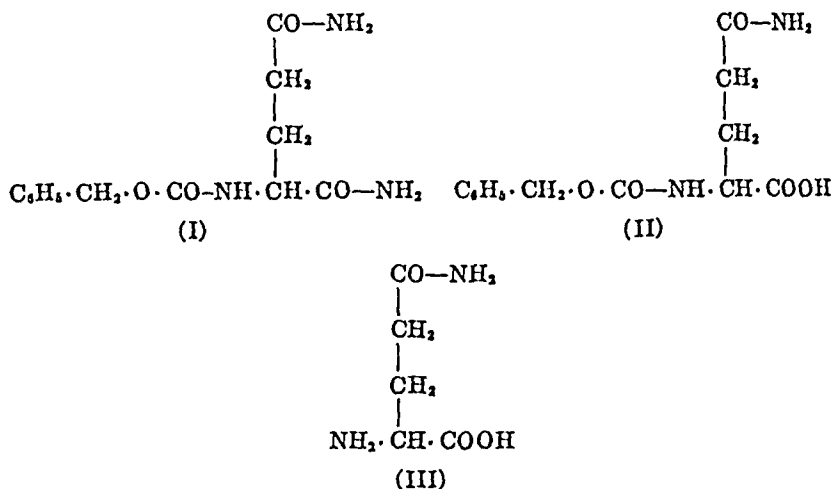
The importance of glutamine in the nitrogen metabolism of plants has been appreciated since the classical studies of Schulze in the last century. The recognition, in recent years, of the rôle of glutamine in the metabolism of animals has conferred upon this substance increased biochemical significance. An excellent survey of the current knowledge concerning the chemical properties and physiological function of glutamine has been provided by Archibald (1).

In the present communication, a method is described for the synthesis of *l*(+)-glutamine. This method is intended to supplement two excellent procedures currently available for the preparation of this substance. The first of these, the isolation of glutamine from plant materials, has been described by Vickery, Pucher, and Clark (2) and is a modification of the method originally used by Schulze and Bosshard (3). The second procedure is the synthesis described by Bergmann, Zervas, and Salzmänn (4) in which carbobenzoxy-*l*-glutamic acid anhydride is allowed to react with benzyl alcohol to give the α -benzyl ester. Conversion of the γ -carboxyl to the acid chloride, followed by treatment with ammonia, yields N-carbobenzoxy-*l*-glutamine benzyl ester which, on catalytic hydrogenation, yields pure glutamine. Nienburg (5) has described an additional synthesis in which glutamic acid γ -ethyl ester hydrochloride (6) is carbobenzoxyated and, from the resulting product, carbobenzoxyglutamine is obtained by treatment with liquid ammonia; hydrogenation yields glutamine.

The synthesis of glutamine described below takes advantage of the fact that the specificity of papain is adapted to the hydrolysis of amide linkages involving the α -carboxyl of N-acylated *l*-glutamic acid (7), while the γ -amide linkages remain unaffected. Consequently, when carbobenzoxy-*l*-glutamic acid diamide (I) is subjected to the action of cysteine-activated papain, the enzymatic cleavage which ensues is confined to the α -amide group. From the enzymatic digest, carbobenzoxy-*l*-glutamine (II) may be isolated. After careful purification of this substance by recrystallization, removal of the carbobenzoxy residue by catalytic hydrogenation yields *l*(+)-glutamine (III) of high purity. The over-all yield, based on

*These experiments were conducted with the aid of grants from the Rockefeller Foundation and from the Fluid Research Fund of Yale University School of Medicine.

the amount of *l*-glutamic acid used to prepare the diamide, is about 20 per cent of the theory.



The purity of the material thus obtained is indicated by a satisfactory elementary analysis and by the fact that, on addition of Nessler's reagent, no immediate color is noted. On standing, however, color is developed because of the cleavage of glutamine in the strongly alkaline solution. This test, suggested by Archibald (1), provides a rapid and sensitive means of demonstrating the absence or presence of ammonium pyrrolidone carboxylate in preparations of glutamine.

Since the operations in this synthesis do not involve the danger of racemization, optical purity of the glutamine may be expected if the *l*-glutamic acid originally employed is optically pure. The glutamine obtained by the method described in this paper gave $[\alpha]_D^{23} = +6.0^\circ$ (3.6 per cent in water), while the optical activity of the product obtained by Bergmann *et al.* (4) by the synthesis described above was reported to be $[\alpha]_D^{19} = +8.0^\circ$. For this reason, an examination was made of the optical rotation of several glutamine preparations of assured purity.

It should be mentioned that Schulze and Trier (8), in a careful study of a variety of glutamine preparations from plant material, found values for the rotation of this substance which varied between $+5.2^\circ$ and $+9.5^\circ$. After critical evaluation of their data, these authors concluded that the specific rotation of *l*-glutamine at 18–20° lay between $+6.0^\circ$ and $+7.0^\circ$. They also called attention to the fact that the rotation of solutions of glutamine could be raised appreciably by the presence, as an impurity, of small amounts (5 per cent) of *l*-glutamic acid.

Through the kindness of Dr. H. B. Vickery, it was possible to examine

the optical activity of two preparations of pure glutamine isolated from natural sources. The first of these was obtained from beets by the method described earlier (2), and had a nitrogen content of 19.0 per cent. The second preparation, isolated from a rye grass exudate, had been recrystallized from water and had a nitrogen content of 19.16 per cent. It was found that the material from beets gave $[\alpha]_D^{25} = +6.0^\circ$, while the material from the grass exudate gave $[\alpha]_D^{25} = +6.1^\circ$. Furthermore, a preparation of *l*-glutamine synthesized according to the method of Bergmann *et al.*, and which had a nitrogen content of 19.20 per cent, gave $[\alpha]_D^{25} = +6.1^\circ$. The available evidence points, therefore, to the value of $+6.0^\circ$ to $+6.1^\circ$ as being the most probable specific rotation of *l*(+)-glutamine at the temperature and concentration employed in these measurements.

It may be added that the method for the synthesis of glutamine described in this paper may be used also for the synthesis of peptides in which glutamine bears the terminal α -carboxyl group (*e.g.*, *l*-phenylalanyl-*l*-glutamine). This supplements the application of the carbobenzoxy method to the synthesis of glutamine peptides by Melville (9) and Harington and Mead (10).

EXPERIMENTAL

Carbobenzoxy-l-glutamic Acid Diamide—44 gm. (0.3 mole) of *l*-glutamic acid ($[\alpha]_D^{20} = +31.0^\circ$ in *N* HCl) were esterified with 250 ml. of absolute methanol and dry hydrogen chloride gas. The solution was evaporated under reduced pressure to a syrup which was dissolved in 200 ml. of absolute methanol. The treatment with dry hydrogen chloride was then repeated. Removal of the methanol by evaporation under reduced pressure gave a syrup. This was dissolved in 200 ml. of water and 300 ml. of chloroform were added. The mixture was chilled and 5 gm. of magnesium oxide and 18 gm. of carbobenzoxy chloride were added with cooling and shaking. Three further additions of the same amounts of the two reagents were made at 10 minute intervals. 15 minutes after the last addition, the excess of carbobenzoxy chloride was decomposed with 10 ml. of pyridine and the reaction mixture was acidified with 5 *N* hydrochloric acid. The chloroform layer was washed twice with water, then successively with bicarbonate, with dilute hydrochloric acid, and finally again with water. After being dried with sodium sulfate, the solution was concentrated under reduced pressure at 35–40°, yielding a syrup which consisted of carbobenzoxy-*l*-glutamic acid dimethyl ester.

To the syrupy ester there were added 500 ml. of absolute methanol which had been saturated previously with ammonia gas at 0°. The mixture was allowed to stand at room temperature for 3 days, during which time the diamide crystallized, and was then chilled overnight in the cold room.

The crystals were filtered, washed with 200 ml. of cold methanol, and recrystallized from absolute alcohol; m.p., 194–196°. The yield was 55 gm. or 65 per cent of theory.

$C_{11}H_{17}O_4N_3$.	Calculated.	C 55.9, H 6.1, N 15.05
279.3	Found.	" 55.8, " 6.2, " 14.9

Carbobenzoxy-l-glutamine—14 gm. of the diamide were suspended in 150 ml. of M/15 citrate buffer (pH 5.0) and to this mixture there were added 100 ml. of a solution of activated papain containing 1 gm. of papain (prepared according to Grassmann (11)) and 250 mg. of cysteine hydrochloride. The solution was diluted to make the total volume 500 ml., and kept for 4 days at 40°. During this period, the diamide went into solution. A small amount of amorphous material then was filtered off, and the filtrate was concentrated under reduced pressure to a volume of 50 ml. Dilute hydrochloric acid was added to Congo red, whereupon an oily precipitate appeared which crystallized readily. Two recrystallizations from methanol-water were required to obtain completely pure material; m.p. 136–137°. Yield, 6.6 gm. or 47 per cent of the theory.

$C_{13}H_{19}O_5N_3$.	Calculated.	C 55.7, H 5.8, N 10.0
280.3	Found.	" 55.7, " 5.8, " 10.0

Bergmann and Zervas (12) have reported a melting point of 137° for this compound, which they prepared by treatment of natural *l*-glutamine with carbobenzoxy chloride in the presence of bicarbonate.

l-Glutamine—4.5 gm. of the carbobenzoxy compound were dissolved in 25 ml. of methanol and 0.2 ml. of glacial acetic acid, and hydrogenated at atmospheric pressure with palladium black (13) as the catalyst. After 3 hours, the hydrogenation was complete and water was added to dissolve the glutamine which had crystallized out. The catalyst was filtered off, and the filtrate was evaporated to dryness under reduced pressure at a bath temperature of 35–40°. The product was recrystallized from water-ethanol (1:3); m.p. 185–186°. Yield, 1.9 gm. or 82 per cent of the theory.

$C_9H_{15}O_2N_2$.	Calculated.	C 41.1, H 6.9, N 19.2
146.1	Found.	" 41.0, " 6.9, " 19.2
$[\alpha]_D^{25} = +6.0^\circ$ (3.6% in water)		

The optical rotation was determined with a Schmidt and Haensch polarimeter equipped with a General Electric sodium lamp as the light source.

The author is indebted to Dr. H. B. Vickery for valuable criticism concerning this manuscript and for the gift of samples of natural glutamine.

SUMMARY

A method for the synthesis of *l*(+)-glutamine is described, in which carbobenzoxy-*l*-glutamic acid diamide is treated with papain to yield carbobenzoxy-*l*-glutamine. Catalytic hydrogenation of this product yields glutamine of high purity.

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THE METABOLISM OF SULFUR

XXXII. ISOCYSTEINE

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Isocysteine and isocystine were first prepared early in the present century by Gabriel (1) as part of a project whose objective was the determination of the structure of cystine, which had only recently been discovered as a component of the protein molecule. Since the structure of cystine was established by Friedmann (2) shortly thereafter, Gabriel did not report his synthesis till 1905 and 2 years later announced a second method of synthesis of isocysteine (3). There was little further work on isocysteine until 1939, when Schöberl and Braun (4), in an investigation of the lability of the sulfur of cystine and related compounds, made available a new and more convenient method of synthesis of isocysteine by which better yields were obtainable. Chemical studies are limited mainly to investigations of lability of the sulfur of isocystine (4). The compound does not react in the highly specific naphthoquinone sulfonic acid test of Sullivan (5) for cysteine but reacts positively, as does cysteine, in the Fleming reaction.¹

So far as is known to us, no studies of the biochemical behavior of isocysteine have been reported. This compound is of interest not only because of the comparison with the isomeric sulfur-containing compound cysteine, but also because of its β -amino group. The group of β -amino acids is represented in nature chiefly by β -alanine, a constituent of carnosine and pantothenic acid. The present study is concerned with the metabolic behavior of isocysteine in the organism of the rabbit and rat. Observations on the reaction of nitrous acid with isocysteine were also made, since it is known that the sulfur of cystine, the disulfide of the isomeric α -amino acid, cysteine, is readily oxidized to sulfate with this reagent, which is used in the Van Slyke method for amino nitrogen (7).

EXPERIMENTAL

After preliminary trials, the method of Schöberl and Braun (4) was adopted for the synthesis of isocysteine as preferable in ease of manipulation and yield. The isocysteine hydrochloride obtained was not of satisfactory

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¹Unpublished observations of Sullivan cited by Vassel (6).

purity, as evidenced by a low sulfur content (18 per cent). In purification, best results were obtained when small amounts (usually 2 gm.) of the impure isocysteine hydrochloride were used. The isocysteine was dissolved in about 5 ml. of water and this solution was added drop by drop with continuous stirring to 100 ml. of a saturated aqueous solution of mercuric chloride. After standing 8 to 12 hours in a refrigerator, the precipitated mercury mercaptide was filtered off, washed, and dissolved in lukewarm 10 per cent hydrochloric acid. After filtration, the solution was treated with hydrogen sulfide and mercuric sulfide was removed by filtration. The filtrate was then distilled at a pressure of a 2 to 3 mm. of mercury until a thick syrup was obtained. The syrup slowly crystallized on standing in a vacuum desiccator. The crystals were filtered off and washed with acetone and ether. The first lot (15 gm., a pooled sample from a number of runs) was of satisfactory purity when first prepared but, after standing for 6 months in a vacuum desiccator, some decomposition had occurred, as evidenced by slightly low total and sulfhydryl sulfur contents. A second lot (8 gm.) was prepared, purified, and used within a few weeks after preparation in most of the experiments reported. Analysis showed a content of total sulfur of 20.36 per cent (theoretical, 20.34 per cent) by the Carius method, 20.56 per cent of sulfhydryl sulfur as determined by the method of Lavine (8), and 22.90 per cent of chlorine (theoretical, 22.56 per cent).

In attempts to convert isocysteine to isocystine, the yields, as reported by others (4), were poor, so that a small amount only of isocystine was prepared (26.68 per cent of sulfur; theoretical, 26.66 per cent) for use in the establishment of standard curves in the colorimetric determination of disulfides in the urine by a modified procedure of Kassell and Brand (9).

Reaction of Nitrous Acid with Isocysteine—The experimental procedure was the same as that previously described in the study of the reaction of nitrous acid with cystine (7). Control experiments in which the oxidation of cystine was determined were carried out in order to make possible the direct comparison of cystine and isocysteine. The rate and extent of the oxidation of cystine to sulfuric acid agree well with those of previous experiments in this laboratory in which the temperature was slightly lower (25°). The results of two series, presented in Table I, show that the sulfur of isocysteine is oxidized to sulfate by nitrous acid at approximately the same rate and to the same extent as is that of cystine. It was not possible to determine whether the nitrogen liberated in the reaction was in excess of the theoretical for β -amino nitrogen as with cystine, but, in view of our previous observations (7), this would appear probable.

Oxidation of Sulfur of Isocysteine by Rabbit—The general procedures of the metabolic experiments with male rabbits of 2 to 3 kilos of body weight

were essentially those customarily used in similar studies in this laboratory (10). In addition to the determination of the partition of urinary sulfur, urinary nitrogen and creatinine, urinary disulfides were also determined by slight modification of the Kassell-Brand phosphotungstic acid procedure (9) and keto acids by Shacter's unpublished modification of the method of Case (11). Phenylpyruvic acid was used as a standard in the determination of the *K* values in the Evelyn photoelectric colorimeter (12) and the extra sulfur as α -keto acid was calculated on the assumption that any increased elimination of keto acids on the experimental days was occasioned by the deamination of the amino acid fed and that each molecule of keto acid corresponded to 1 atom of sulfur. As a matter of fact, no significant

TABLE I

Oxidation of Sulfur of Cystine and Isocysteine to Sulfate Sulfur by Nitrous Acid at 28°

The amount of the sulfur-containing amino acids used in each experiment was equivalent to 50 mg. of sulfur.

Series	Time	Cystine			Isocysteine		
		BaSO ₄	Sulfur oxidized of total		BaSO ₄	Sulfur oxidized of total	
	<i>hrs.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
A	3	154.5	21.3	42.6	161.5	22.2	44.4
		156.6	21.6	43.2	155.2	21.3	42.6
	6	163.1	22.4	44.8	164.8	22.6	45.2
		162.1	22.2	44.4	167.8	23.0	46.0
B	1.5	143.1	19.7	39.4	126.7	17.4	34.8
		142.6	19.6	39.2	125.7	17.3	34.6
	3	151.2	20.8	41.6	147.1	20.2	40.4
		152.3	20.9	41.8	150.5	20.7	41.4
	6	162.7	22.4	44.8	165.8	22.3	44.6
		163.5	22.5	45.0	169.9	23.4	46.8

changes in the excretion of keto acids following the administration of either cystine, cysteine, or isocysteine were observed.

Schöberl and coworkers (4, 13) have determined isocysteine and isocystine in pure solution by a method which made use of the reduction of phosphotungstic acid by the sulfhydryl group. Since it was desired to determine total sulfhydryl and disulfide sulfur in urine, the procedure of Kassell and Brand (9), in which reduction of phosphotungstic acid is also concerned, seemed most promising, inasmuch as it is possible to correct for the non-sulfur reducing substances which are normally present in urine. The conditions recommended by Kassell and Brand were followed with three exceptions. The determinations of optical density were made with an Evelyn photoelectric colorimeter (filter No. 690), the method being

standardized and calibration curves prepared by the use of solutions of pure cystine and isocystine. The sulfite was added prior to the phosphotungstate reagent, since it was found that this resulted in better duplicate determinations. 15 minutes were allowed for color development rather than the 8 minute period recommended by Kassell and Brand. This was necessary because, under the conditions used, the reaction of isocystine was found to be less rapid than that of cystine. When cystine and isocystine were added to rabbit urine, recoveries of 95 ± 2 per cent could be obtained. Since the amounts of isocystine excreted after the administration of isocystine were so large, the accuracy of the method was considered satisfactory.

The urines of the experimental periods were also tested qualitatively by various reactions which indicate thiol and disulfide derivatives. Positive ammonia-nitroprusside and cyanide-nitroprusside tests indicate the presence of thiol and disulfide groups respectively, but the tests are not specific beyond this. A positive Sullivan naphthoquinone sulfonic acid test shows the presence of cysteine or cystine, according to the conditions used. The Fleming test, as modified by Vassel (6), is believed to be specific for a thiol and a primary amine group separated from each other by two $-\text{CH}_2-$ (methylene) groups as in cysteine or isocystine. We have confirmed the observation of Sullivan (6) that isocystine (and isocystine after reduction) gives a positive Fleming-Vassel test.

The experimental data are presented in Table II, in which the distribution of extra urinary sulfur following the oral and subcutaneous administration of isocystine (as the hydrochloride) and of cystine and cysteine is given. When cysteine or cystine was administered, the greater part of the sulfur of the amino acid was recovered in the urine in oxidized form (sulfate sulfur), indicating that the organism of the rabbit can effectively metabolize the sulfur of these compounds when ingested in moderate amounts, as shown by previous experiments in our own and other laboratories. The urines of the experimental days gave weakly positive cyanide-nitroprusside, Fleming-Vassel, and Sullivan tests, evidence that the extra disulfide sulfur was due to the excretion of small amounts of cystine.

In contrast to these findings, the experiments in which isocystine was fed failed to show significant oxidation of the sulfur. Less of the total sulfur fed was recovered as urinary sulfur (29 to 37 per cent as contrasted with 53 to 92 per cent in the cysteine experiments) and the extra organic sulfur fraction of the urine was greatly increased. In two of the experiments in which isocystine was fed, no increase in the oxidized sulfur resulted and 96 to 108 per cent of the extra sulfur was excreted as organic sulfur. When isocystine was injected subcutaneously, although the recovery of total sulfur was much greater (75 to 98 per cent), from 84 to

97 per cent of this sulfur was present in the urine as organic sulfur. The failure of oxidation of the sulfur of isocysteine, in sharp contrast to the ease of oxidation of cystine sulfur, was unexpected, since earlier work with

TABLE II

Excretion of Extra Urinary Sulfur after Administration of Cysteine and Isocysteine

The compounds were administered as the hydrochlorides. The second horizontal line of each experiment represents the sulfur excretions in percentages as follows. The values in parentheses under the heading "Total" represent the percentage of sulfur administered excreted as extra total sulfur; those under the headings "Sulfate" and "Organic," the percentages of the total extra sulfur excreted as oxidized (sulfate) and organic sulfur respectively; and those under the headings "Disulfide" and "Keto acid," the percentages of the extra organic sulfur present as disulfide and keto acid respectively.

Rabbit and experiment No	Substance administered	Sulfur	Extra sulfur of urine				
			Total	Sulfate	Organic	Disulfide	Keto acid
		mg	mg.	mg	mg	mg	mg.
1-1	Cysteine, oral	333	231 (69)	195 (85)	36 (15)		
1-2	Isocysteine, oral	333	97 (29)	-8 (-8)	105 (103)		
1-3	" subcutaneous	150	147 (98)	23 (16)	124 (84)	100 (80)	3 (2)
3-1	Cysteine, oral	333	264 (79)	210 (80)	54 (20)	3 (6)	2 (<1)
3-2	Isocysteine, oral	300	112 (37)	38 (34)	74 (66)	56 (75)	3 (1)
3-3	" subcutaneous	150	114 (76)	8 (7)	106 (93)	86 (81)	3 (2)
4-1	Cysteine, oral	333	176 (53)	147 (84)	29 (16)	10 (35)	1 (<1)
4-2	Cystine "	333	250 (75)	210 (84)	40 (16)	3 (7)	1 (<1)
4-3	Isocysteine, oral	300	101 (34)	4 (4)	97 (96)	77 (79)	2 (<1)
4-4	" subcutaneous	150	117 (78)	4 (3)	113 (97)	88 (78)	5 (3)

thiolactic and thioglycolic acid had shown considerable oxidation of the sulfur of thiol groups substituted on aliphatic fatty acid chains (14).

Determination of the disulfide groups of the urine showed that from 75 to 81 per cent of the extra sulfur of the urine after isocysteine administration was excreted in this form. This was further checked by qualitative tests of the urine. The ammonia-nitroprusside test was negative, demonstrating the absence of cysteine or isocysteine. The Sullivan test was completely

negative and the cyanide-nitroprusside and Fleming-Vassel tests were strongly positive. This indicated the absence of cystine and the presence of a disulfide, presumably isocystine, since the presence of cystine was ruled out by the negative Sullivan test. That significant deamination of the isocystine had not occurred is shown by the determinations of the keto acids. The changes in the keto acid values after isocystine were so slight as to be almost within the error of the determination. The marked increase in disulfide sulfur, presumably isocystine sulfur, and the absence of any notable increase in keto acid excretion suggest strongly that isocystine is not catabolized readily either by oxidative deamination or by oxidation of the sulfur of the molecule.

The relation between the amino and thiol groups of cysteine and isocystine is the same; *i.e.*, in both these compounds the amino and thiol groups are attached to adjacent carbon atoms. In cysteine, the amino group is α to the carboxyl group, while in isocystine the amino group is in the β position. It is possible that in isocystine as well as in cystine (15) the oxidation of the sulfur to sulfate must be accompanied or preceded by oxidative deamination. If the resistance of β -alanine and isoserine to deamination in tissue slice experiments (16) is a characteristic of the entire group of β -amino acids, the deamination of isocystine might be difficult and this failure of deamination might be associated with the failure of oxidation of the sulfur in the animal body. As further indirect evidence of the metabolism of β -amino acids, Schofield, in unpublished results from this laboratory, has shown that, while α -alanine is a readily available source of glycogen in the fasted young white rat, no glycogen is formed under the same conditions when β -alanine is fed. Similarly, in contrast to serine, isoserine is a poor source of glycogen. β -Alanine does not give rise to urinary glucose in the phlorhizinized dog (17), although isoserine is stated to be gluconeogenic under the same conditions (18).

The fate of that fraction of the sulfur administered orally as isocystine, which failed to be excreted as extra sulfur in the urine, is not indicated by these experiments. The bacterial flora may play a rôle in the destruction of sulfur-containing compounds in the intestinal tract (19), but it seems improbable that if this were the chief factor so consistent and so marked a difference between cysteine and isocystine would have been observed. Delayed absorption of isocystine might afford more opportunity for the activity of microorganisms. It may be noted that Schofield in further unpublished experiments has noted a definitely lower rate of absorption of β -alanine and isoserine from the intestine in comparison with the isomeric α -amino compounds, alanine and serine.

Since isocystine was shown to be physiologically inert and to be excreted in large part unchanged in the urine of rabbits to which it was administered,

it was considered improbable that it would favorably influence the growth of young white rats fed a cystine-deficient diet. A short series of experiments, limited by the supply of isocysteine available, was undertaken. The basal cystine-deficient diet was of the usual type, in which the protein was furnished by casein at a 5 per cent level. Cystine and isocysteine were added as supplements at a level of 0.5 per cent. The paired feeding method was employed, the food intake being determined in each group by the amount of food consumed by the animal receiving isocysteine. The rats ate the isocysteine diets poorly. This may have been due to the characteristic and (to the authors) unpleasant taste of the compound. In the paired feeding period, two rats receiving cystine gained 2.8 and 2.6 gm. respectively in 22 days, while their paired control mates receiving isocysteine lost 11.5 and 2.0 gm. In a subsequent 19 day period of unrestricted feeding, the same cystine-fed rats gained 20.3 and 15.1 gm., while the two receiving isocysteine lost 3.1 gm. and gained 0.1 gm. respectively. Although the food intakes were admittedly unsatisfactory, the results offered no evidence of the utilization of isocysteine for growth.

SUMMARY

1. The sulfur of isocysteine was oxidized to sulfate by nitrous acid at approximately the same rate and to the same extent as was the sulfur of cystine.

2. When isocysteine was administered orally or subcutaneously to rabbits, there was no significant increase in the oxidized sulfur of the urine. All of the extra urinary sulfur appeared in the organic sulfur fraction. This extra organic sulfur was mainly disulfide sulfur. The increased excretion of disulfide sulfur was not due to the presence of cystine (negative Sullivan reaction). This negative evidence together with a positive Fleming-Vassel test (after reduction) suggests that isocysteine is excreted as isocystine and that extensive oxidation of the sulfur has not occurred.

3. The relation of these observations to the problem of the metabolism of β -amino acids is discussed.

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MICROBIOLOGICAL DETERMINATION OF METHIONINE IN PROTEINS AND FOODSTUFFS*

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Studies on the metabolism of methionine in the animal body necessitate the use of a reliable method for the determination of methionine in a variety of materials such as proteins, blood, and urine. The usual methods for the estimation of methionine have been chemical in nature and are often insensitive and are in some cases not entirely specific (1-7). Since microbiological methods have been successfully applied to the determination of a number of amino acids in this and other laboratories, such methods seemed to offer the best approach for the analysis of methionine in crude materials.

The requirement of methionine for the growth of certain lactic acid-producing bacteria has been shown independently by several investigators. Shankman *et al.* (8, 9) and Hegsted (10) reported that methionine is essential for the growth of *Lactobacillus arabinosus* 17-5. Kuiken *et al.* (11, 12) and McMahan and Snell (13) also showed that methionine was necessary for maximum growth, although it was not classified as an "essential" amino acid for this organism.

Snell and Guirard (14) studied the qualitative requirements of *Streptococcus faecalis* R and reported that methionine was not essential for this organism. However, Greenhut *et al.* (15) noted that very little growth occurred with this organism when methionine was omitted from the medium and suggested that it could be used for the assay of methionine. Stokes *et al.* (16) have used *Streptococcus faecalis* for the assay of nine essential amino acids, including methionine.

Dunn *et al.* (17, 18) concluded that *Leuconostoc mesenteroides* PD-60 and *Lactobacillus fermenti* 36 could be used to determine methionine in protein hydrolysates. Methionine has also been reported to be essential for *Strep-*

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tococcus lactis L-103 (19) and stimulatory to the growth of *L. casei* BC-1 (20) and *L. delbrückii* LD-5 (21).

It can be seen that there is no complete agreement on the essential nature of methionine in the media of *Lactobacillus arabinosus* and *Streptococcus faecalis*. Several reasons for this disagreement have become evident and are discussed in the present report.

EXPERIMENTAL

Stock cultures of *Lactobacillus arabinosus* 17-5, *Streptococcus faecalis* R, and *Leuconostoc mesenteroides* PD-60¹ were maintained and the inocula were prepared as described by Schweigert *et al.* (22), Teply and Elvehjem (23), and Dunn *et al.* (17), respectively.

The compositions of the basal media and the method of preparation of the stock solutions are presented in Table I. The levels of the constituents were revised slightly to produce more reliable methionine standard curves and to permit the use of similar media for all three microorganisms. The essential differences in the amino acid composition relate to the use of leucine free from methionine (usually *dl*-leucine) in the media of all three organisms and the reduction of the level of alanine for *Leuconostoc mesenteroides* used by Dunn *et al.* (17) more nearly to that used by Hac *et al.* (24). Since pyridoxamine is preferred for *Streptococcus faecalis* (25) and a rather high level of pyridoxine has been used by Dunn *et al.* (17) for *Leuconostoc mesenteroides*, both were used in the stock vitamin solution. However, pyridoxine could probably be eliminated from the media when pyridoxamine is used, since the latter is more active for these organisms. Typical standard curves for *l*-methionine obtained with *Lactobacillus arabinosus*, *Streptococcus faecalis*, and *Leuconostoc mesenteroides* are shown in Fig. 1.

Toennies (26) reported that the methionine and tryptophane in casein could be destroyed by oxidative treatment with hydrogen peroxide. Assays of such a preparation of casein with rats by Bennett and Toennies (27) and microbiological assays in this laboratory indicate that the casein preparation is very low in methionine and tryptophane. The authors have used an acid hydrolysate of casein treated in a similar manner for some of the methionine assays reported here.

Synthetic *dl*-methionine was used for the standard curves in this study. The activity of the two optical isomers was checked with *Lactobacillus arabinosus* as the test organism. Since *d*(+)-methionine was inactive and *dl*-methionine had half the activity of *l*(-)-methionine, the use of *dl*-me-

¹ These organisms are obtainable from the American Type Culture Collection, Georgetown University School of Medicine, Washington, D. C. *Streptococcus faecalis* R is also known as *Streptococcus lactis* R.

TABLE I
Composition of Basal Media*

Constituents	<i>Lactobacillus</i> <i>arabinosus</i> (Schweigert et al. (22))	<i>Streptococcus</i> <i>faecalis</i> (Greenhut et al. (15))	<i>Leuconostoc</i> <i>mesenteroides</i> (Dunn et al. (17))
	mg. per tube	mg. per tube	mg. per tube
Amino acids†			
Oxidized casein hydrolysate‡	20 (0.2 ml.)	50 (0.5 ml.)	50 (0.5 ml.)
l(+)-Glutamic acid	4	4	4
l-Asparagine	4	4	4
l(+)-Lysine monohydrochloride + H ₂ O	2	2	2
dl-Threonine	2	2	2
dl-Valine	2	2	2
dl-Isoleucine	2	2	2
dl-Alanine	2	1	2
l(-)-Cystine	1	2	2
dl-Leucine§	2	2	2
dl-Phenylalanine	1	1	1
l(+)-Arginine hydrochloride	0.5	0.5	1
l(+)-Histidine hydrochloride + H ₂ O	0.5	0.5	1
l(-)-Tyrosine	0.5	1	1
dl-Tryptophane	0.5	1	1
Glycine		0.2	1
dl-Serine		0.5	2
l(-)-Proline			0.5
dl-Methionine*	2	1	2
Glucose	200	200	200
Sodium acetate	200		200
" citrate + H ₂ O		250	
Purines and pyrimidines	(0.1 ml.)	Same as <i>L.</i> <i>arabinosus</i>	Same as <i>L.</i> <i>arabinosus</i>
Adenine sulfate + 2H ₂ O	0.1	" "	" "
Guanine hydrochloride + 2H ₂ O	0.1	" "	" "
Uracil	0.1	" "	" "
Xanthine		0.1 (0.1 ml.)	0.1 (0.1 ml.)
Salts A	(0.05 ml.)		Same as <i>L.</i> <i>arabinosus</i>
KH ₂ PO ₄	5		" "
K ₂ HPO ₄	5	50 (0.1 ml.)	" "
Salts B	(0.05 ml.)	Same as <i>L.</i> <i>arabinosus</i>	" "
MgSO ₄ ·7H ₂ O	2	" "	" "
NaCl	0.1	" "	" "
FeSO ₄ ·7H ₂ O	0.1	" "	" "
MnSO ₄ ·4H ₂ O	0.1	" "	" "

TABLE I—Concluded

Constituents	<i>Lactobacillus arabinosus</i> (Schweigert <i>et al.</i> (22))	<i>Streptococcus faecalis</i> (Greenhut <i>et al.</i> (15))	<i>Leuconostoc mesenteroides</i> (Dunn <i>et al.</i> (17))
	γ per tube (0.1 ml.)	mg. per tube Same as <i>L.</i> <i>arabinosus</i>	mg. per tube Same as <i>L.</i> <i>arabinosus</i>
B-vitamins			
Thiamine hydrochloride	5	" "	" "
Riboflavin	5	" "	" "
Niacin	10	" "	" "
<i>dl</i> -Calcium pantothenate	5	" "	" "
Pyridoxamine dihydrochloride	12	" "	" "
Pyridoxine hydrochloride	50	" "	" "
<i>p</i> -Aminobenzoic acid	5	" "	" "
Biotin	0.01	" "	" "
Folic acid	0.1	" "	" "
Choline chloride	25	" "	" "
<i>i</i> -Inositol	25	" "	" "

Stock solutions were prepared in the concentrations indicated above. Several ml. of HCl and H₂SO₄ were added to Salts B to prevent precipitation. The purines and pyrimidines were dissolved in hot HCl. Xanthine was dissolved in NH₄OH. The B vitamins were dissolved by heating in water. New vitamin solutions were prepared every month. Convenient volumes for all solutions were 500 ml. They were stored under toluene in a refrigerator. The amino acids were ground with a mortar and pestle and dissolved in a few ml. of dilute HCl prior to each assay.

* Methionine is omitted. The numbers in parentheses refer to the volumes of stock solution used.

† Based upon inactivity of the unnatural *d* isomers.

‡ Prepared as described in text; used in place of synthetic amino acids when cystine and tryptophane are added. Proline should also be used for *Leuconostoc mesenteroides*.

§ Preferable to *l*(+)-leucine. Must be free of methionine as is indicated in the text.

|| Not absolutely essential in the medium.

thionine as a standard was justified. The *l*-methionine activity of several related compounds is shown in Table II.

All samples used in this study were ground and thoroughly mixed to assure homogeneity prior to analysis for nitrogen and methionine. The meat samples were stored in a refrigerator at -4° .

The protein samples were hydrolyzed in the autoclave with 2 N HCl at 15 pounds pressure for 5 to 10 hours. The assay values did not increase when the strength of the acid was increased. The rate of liberation of methionine is compared with the α -amino nitrogen liberation (micro nitrous acid method of Van Slyke) in Figs. 2, 3, and 4.

The assay procedure is similar to that reported by Schweigert *et al.* (22). Typical results obtained with different assay levels and with the three

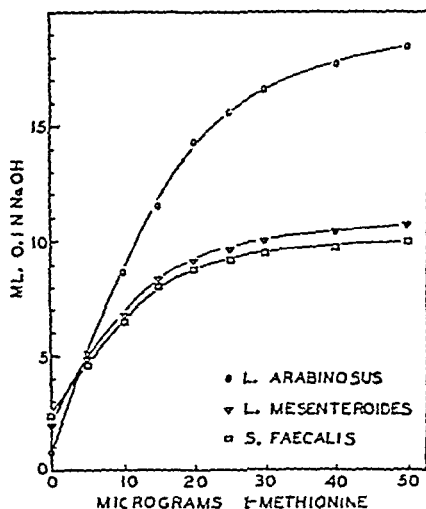


FIG. 1. Typical methionine standard curves obtained with *Lactobacillus arabinosus* 17-5, *Streptococcus faecalis* R, and *Leuconostoc mesenteroides* PD-60.

TABLE II

l(-)-Methionine Activity of Various Compounds and Leucine Contamination Tested with Lactobacillus arabinosus

Compound*	Per cent† activity
<i>dl</i> -Methionine	49.5
<i>d</i> (+)-Methionine	0.1
<i>dl</i> -Homocystine	0.05
" + choline chloride	0.03
<i>l</i> (-)-Cystine	0.0
<i>l</i> (+)-Cysteine	0.0
Glutathione	0.0
<i>l</i> (+)-Leucine (different sources)	
Sample 1	2.1
" 2	2.0
" 3	0.2
" 4	0.2
<i>dl</i> -Leucine (different sources)	
Sample 1	0.2
" 2	0.03
" 3	0.05
" 4	0.0†

* Assayed at levels of 0.5 to 5 mg per tube.

† Average of two assays.

‡ All values are based upon this sample of *dl*-leucine, which was used in the basal medium.

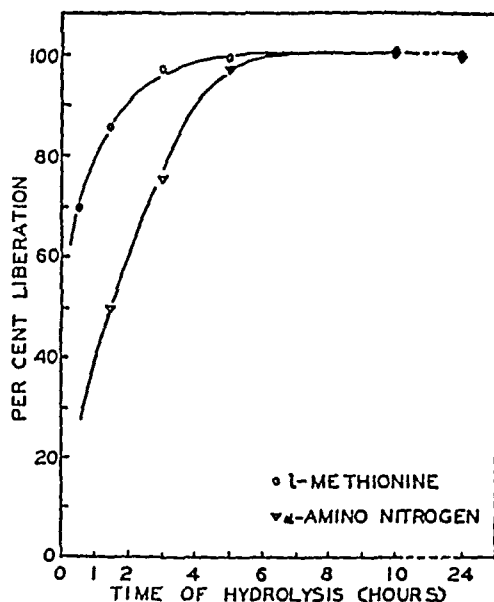


FIG. 2. Effect of time of hydrolysis on the rate of methionine, and α -amino nitrogen liberation in casein. 0.3 gm. samples were autoclaved with 2 N HCl.

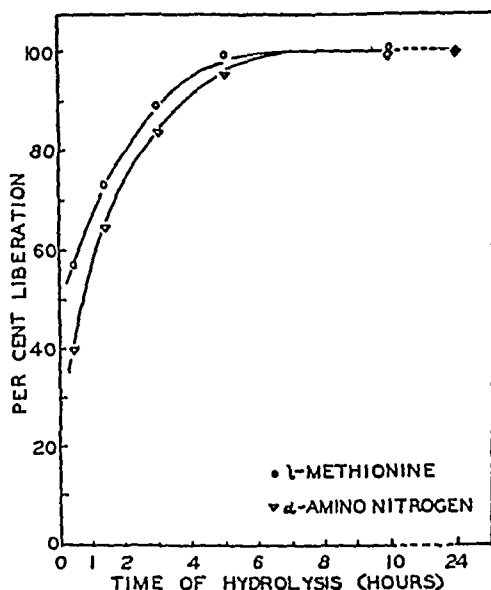


FIG. 3. Effect of time of hydrolysis on the rate of methionine, and α -amino nitrogen liberation in pork muscle. 1 gm. samples were autoclaved with 2 N HCl.

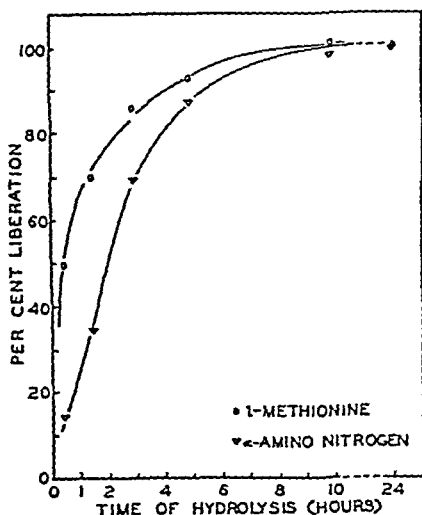


FIG. 4. Effect of time of hydrolysis on the rate of methionine and α -amino nitrogen liberation in raw soy bean oil meal. 1 gm. samples were autoclaved with 2 N HCl.

TABLE III

l-Methionine Content of Soy Bean Oil Meal Determined at Different Assay Levels with Three Different Organisms

		<i>Lactobacillus arabinosus</i>				<i>Streptococcus faecalis</i>				<i>Leuconostoc mesenteroides</i>				
Amount of sample added per tube		0.1 N NaOH titration	<i>l</i> -Methionine per tube	<i>l</i> -Methionine in sample	Amount of sample added per tube		0.1 N NaOH titration	<i>l</i> -Methionine per tube	<i>l</i> -Methionine in sample	Amount of sample added per tube		0.1 N NaOH titration	<i>l</i> -Methionine per tube	<i>l</i> -Methionine in sample
mg.	ml.	γ	per cent		mg.	ml.	γ	per cent		mg.	ml.	γ	per cent	
0.25	1.82	1.9	0.76		0.5	4.30	4.0	0.80		0.5	4.45	3.9	0.78	
0.5	4.50	4.2	0.84		1.0	5.90	8.2	0.82		1.0	6.25	7.9	0.79	
1.0	7.52	8.3	0.80		2.0	8.05	16.3	0.81		2.0	8.40	15.9	0.80	
1.5	10.05	12.2	0.81		3.0	9.03	24.6	0.81		3.0	9.60	24.9	0.83	
2.0	11.55	16.3	0.81		4.0	9.50	32.0	0.80		4.0	10.05	32.6	0.81	
2.5	13.80	21.0	0.83		5.0	9.80	38.0	0.76		5.0	10.30	41.0	0.81	
Average				0.81+					0.80+					0.81+

different organisms are shown in Tables III and IV. The methionine content of some proteins and foodstuffs is shown in Table V.

DISCUSSION AND RESULTS

The standard curves obtained with *Lactobacillus arabinosus*, *Streptococcus faecalis*, and *Leuconostoc mesenteroides* indicate that all three organisms can be used for the assay of methionine. The maximum growth obtained with *Lactobacillus arabinosus* is greater than that with the other two organisms and is similar to the growth obtained by Shankman *et al.* (9) and Schweigert *et al.* (22) with other amino acids. It is of interest to note greater growth response to smaller amounts of methionine when *Lactobacillus arabinosus* was used as the test organism. A suitable assay range

TABLE IV

*Reproducibility of l-Methionine Content Determined with Different Organisms**

		Per cent l-Methionine									
		<i>Lactobacillus arabinosus</i>				<i>Streptococcus faecalis</i>			<i>Leuconostoc mesenteroides</i>		
		Assay No.				Assay No.			Assay No.		
		1	2	3	Average	1	2	Average	1	2	Average
Proteins	Casein	3.10	3.18	3.15	3.14	3.00	3.20	3.1	3.20	3.10	3.15
	Egg albumin	3.20	3.00	3.10	3.10	3.20	3.15	3.17	3.33	3.50	3.41
	Gelatin	1.05	0.99	1.10	1.05	1.03	1.10	1.06	1.01	0.99	1.00
	Pepsin	1.40	1.32	1.46	1.39	1.55	1.35	1.45	1.52	1.48	1.50
Foodstuffs	Pork muscle	0.65	0.60	0.69	0.65	0.64	0.60	0.62	0.62	0.72	0.67
	“ liver	0.55	0.58	0.60	0.58	0.58	0.60	0.59	0.61	0.59	0.60
	Soy bean oil meal	0.81	0.80	0.75	0.79	0.75	0.80	0.77	0.75	0.81	0.78
	Brewers' yeast, dried	0.90	0.85	0.88	0.88	0.80	0.85	0.82	0.87	0.83	0.85

* The assays with each organism were made on two different hydrolysates of the samples. Assay 1 with *Lactobacillus arabinosus* was made by the use of a medium which contained oxidized casein plus tryptophane and cystine. In all of the other assays synthetic amino acids were used in the medium.

with this organism is from 0 to 20 γ of l-methionine per tube, as compared to 0 to 50 γ with *Streptococcus faecalis* and *Leuconostoc mesenteroides*.

The acid production with *Streptococcus faecalis* was approximately twice that obtained by Stokes *et al.* (16); the higher percentage of glucose and the use of citrate buffer rather than acetate in the present study probably account for this difference. Stokes, however, indicated that additional glucose would not increase the acid production appreciably. Previous work in this laboratory by Teply and Elvehjem (23), confirmed by the present workers, showed that the acid production obtained with *Streptococcus faecalis* is greater when citrate is used as the buffer instead of acetate.

In the early work with *Streptococcus faecalis*, many of the methionine standard curves showed irregularities in the middle portion of the curve.

Similar results were reported by Stokes in the development of the method for lysine with the same organism, although no difficulty with methionine was reported. These workers indicated that increasing the levels of the

TABLE V
Methionine Content of Proteins and Foodstuffs

		Per cent nitrogen* (Kjeldahl)	Per cent l-Methionine†	Per cent l-methionine in protein‡	Per cent methionine N in protein N	Values in literature	
						Microbiological, Stokes and Guinness (16)	Chemical, Block and Belling (23, 29)
Proteins	Casein	14.2	3.13	3.40	1.99	2.65‡	3.5‡
	Lactalbumin	15.3	2.48	2.59	1.52		2.8‡
	Egg albumin	11.6	3.21	4.43	2.60	4.1‡	5.0‡
	Fibrin (beef blood)	14.4	2.37	2.64	1.55		2.6‡
	Gelatin	16.3	1.04	1.02	0.66	0.59‡	1.0‡
	Keratin (beef toes)	14.7	1.03	1.18	0.63		
	Pepsin	15.0	1.45	1.54	0.90		1.5‡
	Wheat gluten	13.2	1.56	1.83	1.10		
	“ gliadin	13.7	1.56	1.83	1.07		1.5‡
	Edestin	15.7	2.35	2.40	1.41		1.9‡
Foodstuffs	Pork muscle	2.96	0.56	3.03	1.73		3.0‡
	“ liver	2.45	0.59	3.86	2.26		
	Beef heart	2.90	0.56	3.09	1.82		3.2‡
	“ kidney	2.32	0.28	1.93	1.13		2.8‡
	“ brain	1.64	0.26	2.55	1.49		3.0‡
	Condensed fish solubles	3.42	0.38	1.77	1.39		
	Soy bean oil meal	7.03	0.78	1.77	1.31	0.84‡	1.9‡
	Linseed “ “	6.10	0.84	2.20	1.29	0.81‡	3.0‡
	Alfalfa	2.36	0.21	1.43	0.84	0.15‡	
	Wheat	2.00	0.32	2.53	1.48	1.20‡	3.0‡
	Rolled oats	1.40	0.36	4.12	2.40		
	Yellow corn	1.34	0.30	3.57	2.09		
	White “	1.37	0.26	2.98	1.75		
	Brewers' yeast, dried	9.00	0.85	1.51	0.89	1.37‡	1.37‡

* Average of two determinations.

† Average of three or more assays.

‡ Calculated to 16 per cent N.

§ Per cent in dried material.

purine bases and vitamins eliminated the irregularities. The difficulties were eliminated in our work when the levels of some of the vitamins were increased and when pyridoxamine was used in the basal medium (25).

The typical standard curve obtained with *Leuconostoc mesenteroides* was

similar to that of *Streptococcus faecalis*. When an acid hydrolysate of oxidized casein (26) plus added cystine and tryptophane was substituted for crystalline amino acids in the basal medium, the growth response was similar to that obtained with the synthetic medium for *Lactobacillus arabinosus* and *Streptococcus faecalis*. The response with *Leuconostoc mesenteroides* was not uniform until proline was also added to the hydrolysate.

The methionine standard curves that have been reported by other investigators with various organisms (15-17) show rather high acid production when methionine is omitted from the medium. Several of these workers used *l*(+)-leucine instead of synthetic *dl*-leucine in their basal media. It has been found that the commercial *l*(+)-leucine that has been used in this laboratory frequently contained some methionine. The methionine assay of several leucine samples (Table II) showed that *dl*-leucine was generally free of methionine and was used, therefore, in our basal media. Similar contamination of leucine by methionine has been noted by Baernstein (4). Fox (30) has described a chemical method of removing methionine from leucine.

Table II shows the methionine activity of various compounds tested with *Lactobacillus arabinosus*. The *d* isomer is practically inactive and *dl*-methionine is one-half as active as *l*(-)-methionine. Homocystine, a mixture of homocystine and choline, cystine, cysteine, and glutathione were devoid of methionine activity. Similar results were reported by Stokes *et al.* (16, 31) with *Streptococcus faecalis*.

Results obtained with the studies on hydrolysis showed that methionine was liberated at a more rapid rate than the total α -amino nitrogen from casein, pork muscle, and soy bean oil meal. This increase may possibly be due to the utilization by *Lactobacillus arabinosus* of peptides of methionine which were liberated sooner than the amino acids *per se*. The liberation of methionine was essentially complete after 5 hours hydrolysis in the case of casein and pork muscle, but soy bean oil meal required about 10 hours for complete hydrolysis. Recoveries of methionine added before hydrolysis averaged 100.6 ± 5 per cent. Liberation at 24 hours was considered to be 100 per cent. Since no destruction of methionine occurred with prolonged hydrolysis, it appears that in general 10 hours hydrolysis is preferred to 5 hours for maximum methionine liberation in all proteins used in this study.

Since the hydrochloric acid used in hydrolysis is neutralized with sodium hydroxide prior to assay, the effect of sodium chloride concentration on the assay values was studied. No inhibitory effects were noted at the concentrations that are present in the neutralized hydrolysates. However, higher salt concentrations (15 mg. of sodium chloride per tube) caused decreased acid production with *Lactobacillus arabinosus* and *Streptococcus*

faecalis. No inhibitory effects were observed with *Leuconostoc mesenteroides* at salt concentrations up to 40 mg. per tube. This is in agreement with the results of Dunn *et al.* (17). The maximum amount that was present in the protein hydrolysates in this study was 1.5 mg. of sodium chloride per tube, which is well below the inhibitory level.

Good agreement in the methionine content of natural materials was obtained with different assay levels with each of the three organisms. The results obtained with soy bean oil meal are shown in Table III. Recoveries obtained with all three organisms at the different assay levels averaged 99.6 per cent, with most values occurring within a 10 per cent range. Consistent values were also obtained upon repeated assay with the three organisms (Table IV).

The average methionine content of a number of proteins and natural foodstuffs is shown in Table V. These values are compared with those reported by Stokes and Gunness (16) and those summarized by Block and Bolling (28, 29). In general our values are slightly higher than the microbiological results of Stokes with the same proteins and lower than many of the chemically determined values summarized by Block and Bolling (28). The per cent methionine (calculated to 16 per cent nitrogen for the purpose of comparison with literature values) in casein, lactalbumin, and egg albumin, for which many literature values are available, according to our results are 3.40, 2.59, and 4.43. The values as summarized by Block (28) are 3.5, 2.8, and 5.0 for the same proteins, respectively. The methionine content of the purified proteins ranged from 1.02 in gelatin to 4.43 in egg albumin. Egg albumin has the highest methionine content of any material analyzed. Among the animal foodstuffs, pork liver was found to be the richest in methionine, while condensed fish solubles were lowest. Rolled oats had the highest methionine content among the foodstuffs of plant origin; alfalfa had the lowest. It is interesting to note that oats and corn were richer than wheat in methionine content. It appears that methionine is quite widely distributed among vegetable and animal proteins.

SUMMARY

Satisfactory standard curves were obtained for methionine with synthetic amino acid media when *Lactobacillus arabinosus* 17-5, *Streptococcus faecalis* R, and *Leuconostoc mesenteroides* PD-60 were used as the assay organisms. A hydrogen peroxide-treated casein hydrolysate was also used successfully.

The methionine content of a number of purified proteins and natural foodstuffs has been determined. The reliability of the assay methods has been established by agreement of the results of different organisms with different assay levels of sample and repeated assay. Good recovery of methionine added prior to hydrolysis of samples was obtained. The me-

thionine content of the proteins analyzed agreed satisfactorily with literature values.

Hydrolysis studies show that methionine tends to be liberated faster than α -amino nitrogen in casein, pork muscle, and soy bean oil meal. Hydrolysis was complete after 5 hours autoclaving with 2 N HCl in the case of casein and pork muscle, and after 10 hours in the case of soy bean oil meal.

The importance of using *dl*-leucine or leucine samples free of methionine in the basal medium is discussed.

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THE CHROMATOGRAPHIC SEPARATION OF BILE ACIDS

I. THE SEPARATION OF CHOLIC AND DESOXYCHOLIC ACID

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The physiological and industrial importance of cholic and desoxycholic acids, the two main constituents of the bile acids, makes very desirable a method for their qualitative separation and, if possible, for their quantitative determination. The difference in their solubilities in various solvents or of the solubilities of the salts used in their preparation (1) is not very marked and causes losses too large to allow more than a rough estimation of their quantities and necessitates relatively large amounts of the starting material for successful operation.

The chromatographic analysis of these acids promised a successful separation. The difficulty of the method consists in the fact that the bile acids are colorless substances, and for the purpose of visual chromatographic separation they have to be converted into colored derivatives.

In the case of the bile acids which contain hydroxyl groups there is the possibility of converting them into colored compounds by esterifying the alcoholic OH groups by means of azoyl chloride (2). Although this method may prove successful, it seems to us more desirable to place the "chromophoric" group on the COOH group for the following reasons. (1) It is well known that the different OH groups of the bile acids react differently on esterification; so that without special precautions there may be formed partly esterified compounds in the case of the dihydroxyl and polyhydroxyl compounds which would complicate the chromatographic picture. (2) By coupling the "chromophoric group" to the carboxylic group, not only bile acids containing alcoholic hydroxyl groups, but also acids devoid of them, like cholenic and polyenic acids, as well as the ketonic derivatives, can be included in the scope of the method. (3) The alcoholic hydroxyl groups are known to cause strong adsorptivity of a compound (3), and therefore it is to be expected that mono-, di-, and polyhydroxyl compounds can be differentiated in the chromatogram of their mixture.

As a suitable chromophoric reagent for the esterification of the COOH group we have chosen the ω -bromo-*p*-methylazobenzene. This substance is relatively simple and, being a derivative of benzyl bromide, possesses a highly reactive bromine atom which reacts under suitable conditions with the sodium salts of acids, giving a yield of 85 per cent or higher of the corresponding ester.

ω -Bromo-*p*-methylazobenzene was prepared by Burns, McCombie, and Scarborough (4) by bromination of 4-methylazobenzene but with very moderate yields. We found that this compound can be prepared with better yields and in a high state of purity by condensation of *p*-aminobenzyl alcohol with nitrosobenzene and the transformation of the hydroxymethyl compound into the corresponding bromine derivative by action of phosphorus tribromide, as described in the experimental part.

For the chromatographic separation of the colored esters we tried first activated alumina, silica gel, calcium carbonate, and magnesium carbonate. The two first were too active and did not allow a proper development of the chromatogram; the third was too weak in its adsorptive properties. MgCO_3 (a commercial preparation of the Corona Chemical Company, Sydney) proved a very satisfactory adsorbent for the colored esters dissolved in a mixture of benzene and petroleum ether (65–80°) 1:2. For the development of the chromatogram we first use a mixture of benzene and petroleum ether 1:1 and then pure benzene. The ester of the cholic acid is adsorbed on top as an orange-colored band; the desoxycholic ester (if in mixture with cholic acid ester) is placed below and is lighter in color. It is possible to develop a narrow, almost white, band between these two bands of esters and with some practice there is no difficulty in a fair separation of the two colored zones, provided that a properly packed column is used so that there are no excessive irregularities in the formation of the bands.

5 per cent of cholic acid in desoxycholic acid and 10 per cent of desoxycholic acid in cholic acid can be easily discerned in the developed chromatogram.

At present we are engaged in extending this separation to a quantitative colorimetric determination of the proportions of the two acids present in the mixture and in the visual separation of the esters by using different chromophoric groups, *e.g.* $\text{C}_6\text{H}_5 \cdot \text{N}=\text{N} \cdot \text{C}_6\text{H}_4 \cdot \text{CO} \cdot \text{CH}_2$ halide.

EXPERIMENTAL

*ω -Hydroxy-*p*-methylazobenzene*—The condensation of *p*-aminobenzyl alcohol with nitrosobenzene in alcoholic solution at room temperature proceeds very slowly and gives very low yields. The same condensation in acetic acid yields mostly a brick-red compound, of a very high melting point, which is sparingly soluble in the usual solvents and is not the expected azo compound. In a mixture of alcohol and acetic acid, however, the condensation proceeds smoothly.

5.85 gm. (0.055 mole) of nitrosobenzene are dissolved in a mixture of 40 ml. of alcohol and 14 ml. of glacial acetic acid with careful heating to 40–50°.

The clear green solution is cooled to 15° and 6.1 gm. of aminobenzyl alcohol¹ are added with continuous shaking in six portions within 5 to 10 minutes. The solution turns reddish brown and the temperature rises to approximately 40°. The mixture is shaken for an additional 15 to 20 minutes, when crystallization sets in and it is left for 3 days at room temperature. After standing for a day in a refrigerator, the crystals are filtered and pressed on a Büchner funnel. A further crop can be obtained by diluting the filtrate with water. After drying in air, the material is extracted with warm ether in a continuous extractor (a brown insoluble material remains in the cone). The ethereal solution is evaporated to a small volume until an abundant crystallization sets in. An equal volume of petroleum ether (40–60°) is added, the mixture is cooled, and the orange crystals are filtered with suction and washed with a mixture of ether-petroleum ether. M.p. 139–140.5°; 8.2 gm. = 78 per cent of the theory. This material is pure enough for the next step. For analysis it was recrystallized from ether-petroleum ether (40–60°); m.p. 141.5°.

26.42 mg. $C_{11}H_{12}ON_2$; 71.12 mg. CO_2 ; 13.82 mg. H_2O
Calculated, C 73.59, H 5.66; found, C 73.41, H 5.85

ω-Bromo-*p*-methylazobenzene²—To 6.4 gm. of 4-hydroxymethylazobenzene (0.03 mole) suspended in 150 ml. of dry benzene, 3.2 gm. (120 per cent of the theory) of PBr_3 dissolved in 35 ml. of dry benzene are added within 10 minutes in five equal portions with constant shaking. The orange-red solution turns dark and a flocculent precipitate settles on the walls. After standing $\frac{1}{2}$ hour at room temperature, with periodical shaking, the reaction mixture is decomposed by addition of water. The benzene is evaporated and the residue is extracted with small portions of ether until the last extract is only slightly yellow in color. The ethereal extracts are collected and evaporated to a very small volume. After addition of double the volume of a low boiling petroleum ether (40–60°) and standing in the refrigerator, the crystals are filtered with suction and washed. Orange-yellow crystalline powder, m.p. 111–112°; yield, 6 gm. = 72 per cent of the theory.

¹ *p*-Aminobenzyl alcohol was prepared according to Fischer and Fischer (5) with the following changes: After removing the azo and azoxy compounds, the filtrate was evaporated under reduced pressure (preferably in a CO_2 stream) and the solid residue was exhaustively extracted with ether. The ethereal solution was dried over Na_2SO_4 , the ether evaporated, and the dark brown residue distilled *in vacuo*. B.p. 169–171°, 11 mm.; m.p. 64.5°. After recrystallization from benzene, the melting point was 65°. Yield, 11 to 12 gm. = 68 to 75 per cent of the theory, from 20 gm. of *p*-nitrobenzyl alcohol (6).

² We propose for this compound the term azyl bromide.

By a further recrystallization from ether-petroleum ether the melting point can be raised to 114.5–115°.

24.5 mg. gave 16.7 mg. of AgBr.

$C_{11}H_{11}N_2$ Br. Calculated, Br 29.1; found, Br 29.0

Preparation of Colored Esters of Cholic and Desoxycholic Acids—To a hot solution of 1 gm. of ω -bromo-*p*-methylazobenzene in 15 ml. of alcohol is added a hot solution of 1.4 gm. of sodium cholate (or 1.3 gm. of sodium desoxycholate) (slightly less than the calculated amount) in 5 ml. of water and the homogeneous mixture is boiled under a reflux for 6 to 8 hours. The alcohol is removed under reduced pressure, and the residue is taken up with benzene and washed with water. After evaporation of the benzene layer under a vacuum, the residue is dried *in vacuo* over P_2O_5 and paraffin. For the purification of the crude orange-red ester 100 mg. are dissolved in a mixture of benzene and petroleum ether (70–80°) 1:2 and passed through a column of 25 to 30 gm. of $MgCO_3$. The height of the filled portion is about 26 to 28 cm., 15 mm. inside diameter. The $MgCO_3$ was dried for 1 to 1½ hours at 120° and sifted to pass a 100 mesh screen but not a 150 mesh screen. The chromatogram was developed by washing with 100 ml. of a mixture of benzene-petroleum ether 1:1 and 150 ml. of benzene. The cholic acid ester remained as an orange-colored band, 90 to 100 mm. long, at the top of the column. The desoxycholic acid ester behaved similarly, but the color of the band was more yellow and the band from the same quantity of material was rather wider, 100 to 120 mm. 8 to 10 mg. of 4-hydroxymethylazobenzene (m.p. 140–140.5°) could be isolated from a yellow band which traveled down the column and could be easily separated from the acid esters by a wide white zone or washed into the filtrate. Into the first filtrate passed an unidentified red-colored material, approximately 5 mg. For the elution of the esters we used a mixture of alcohol and benzene 5:95.

In the same way we separated mixtures (40 to 80 mg.) of cholic and desoxycholic esters in the proportions of 1:1, 1:5, 5:1, 1:10, and 10:1.

In the developed chromatogram there was always an easily perceptible pale band on the border between the cholic and desoxycholic esters.

The chromatogram was eluted and the purity of the material separated was tested by the following procedure. The solution of the ester in alcohol containing benzene was evaporated under reduced pressure, the residue taken up in 1 to 1.5 ml. of alcohol, 1 to 2 drops of 50 per cent KOH were added, and the mixture was refluxed for 1 to 1½ hours. Water was added and the alcohol was removed by repeated evaporation. The strong alkaline solution was acidified and the acids were redissolved by adding a solution of Na_2CO_3 . The mixture was extracted three to four times with ether, until the ether layer was colorless, freed from the dissolved ether by heating,

cooled, and acidified to Congo red. The separated acid was filtered and dried at 120° over P_2O_5 at 8 to 10 mm. for 3 hours. The cholic acid fraction invariably had a melting point of $196-198^{\circ}$; that of desoxycholic acid was $171-172^{\circ}$ (checked as the acetic acid-choleic acid, m.p. 142°).

For the purpose of the chromatographic analysis it is not necessary to submit the crude mixture after esterification to any purification, as the esters of the cholic and desoxycholic acids are much more strongly adsorbed on $MgCO_3$ than are the contaminants. The contaminants travel down the column or pass into the filtrate.

SUMMARY

Cholic and desoxycholic acids were transformed into colored esters by permitting their sodium salts to react with ω -bromo-*p*-methylazobenzene.

The yellow- to orange-colored esters were separated in a chromatogram by adsorption on magnesium carbonate.

The orange-colored cholic acid ester is more strongly adsorbed and is separated in the developed chromatogram from the lighter colored desoxycholic acid ester by a white band.

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PARTIAL OXIDATION OF HYODESOXYCHOLIC ACID*

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In the course of experiments leading to the synthesis of various steroid hormones it became desirable to study the use of a 6-hydroxy bile acid as an intermediate for the introduction of unsaturation α to a ketone group at C_3 . This reaction had previously been described by Marker and Krueger (1) who prepared progesterone from 3,6-diacetoxy-20-ketopregnane. None of the intermediates were isolated and the authors made no mention of the yield obtained. Several experiments were carried out, therefore, on the hydrolysis of methyl diacetoxyhyodesoxycholate with from 0.05 to 0.15 N NaOH in 85 per cent ethanol. These failed to reveal any evidence for differential hydrolysis of the acetoxy groups.

In general a hydroxyl group at position 3 in the steroid nucleus is more resistant to oxidative attack by CrO_3 than a hydroxyl group attached to Rings B or C. Thus the hydroxyl groups of cholic acid are dehydrogenated by CrO_3 in the order C_7 , C_{12} , C_3 (2-4); the C_6 -OH group of hyodesoxycholic acid is more readily oxidized than the C_3 -OH (5); and a hydroxyl group at C_{11} is more easily oxidized than the C_7 -OH (6, 7). However, a similar generalization cannot be made for other oxidizing agents. In particular the useful method of Oppenauer (8), in which an alcohol is dehydrogenated to the carbonyl derivative by an aluminum alcoholate in the presence of a hydrogen acceptor such as acetone or cyclohexanone, when applied to saturated polyhydroxy steroids results in preferential dehydrogenation of the C_3 -hydroxyl. Thus methyl cholate (9), methyl desoxycholate (9), 3(α),20(α)-pregnandiol,¹ 3,7-dihydroxy-12-acetoxy-20-ketopregnane (10), and 3,12-dihydroxy-20-keto-21-acetoxypregnane (11) yield the 3-keto derivative upon Oppenauer oxidation. The oxidation of the C_3 -hydroxyl is, of course, much more readily and completely accomplished when a double bond is in the vicinity and with such compounds partial oxidation is possible even when the other OH group is primary (11). The ease of oxidation of the C_3 -OH is explicable in steric terms, since the C_7 -hydroxyl in either α or β configuration can easily form the intermediate alcoholate, whereas the

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¹Gallagher, T. F., unpublished observation.

other nuclear hydroxyl groups are hindered by the folding of the fused ring system and its substituents. A single exception to this conclusion appeared possible in the case of a hydroxyl group at C₆. An OH group at this position is less hindered than others attached to Rings B or C, and might be oxidized by Oppenauer's procedure. A preliminary experiment in which methyl hyodesoxycholate in benzene solution was heated under a reflux with aluminum *t*-butoxide and cyclohexanone for 15 hours led to the isolation of methyl 3,6-diketoallocholanate, indicating that the 6-hydroxyl group could be oxidized by this method and that in the course of the reaction isomerization of the cholane structure to the sterically more favorable allocholane derivative had taken place. It was desirable then to determine whether there existed a difference in the rate of oxidation of the hydroxyl groups at positions 3 and 6 sufficient for the isolation of the partially oxidized monoketohydroxy acid.

The Oppenauer oxidation of methyl hyodesoxycholate was investigated and it was possible to isolate the hitherto undescribed 3-keto-6-hydroxycholanolic acid from the reaction products. With this material in hand the partial hydrolysis of methyl diacetoxhyodesoxycholate was reinvestigated and it was found possible to prepare 3-keto-6-hydroxycholanolic acid by oxidation of the products of partial saponification. We were thus able to confirm the experience of Marker and Krueger and establish that the C₃-acetox group is more easily hydrolyzed than the C₆-acetox group, although the yields obtained were small.

The initial aim of the investigation, namely the conversion of a 3-keto-6-hydroxy bile acid to the α,β -unsaturated keto derivative was accomplished by heating methyl 3-keto-6-*p*-toluenesulfoxycholanate with collidine. The product, methyl 3-keto- Δ^4 -cholanate, was obtained in good yield from the reaction. The tosyl ester is somewhat more stable than was expected, since 3 hours refluxing with pyridine resulted in the recovery of over 55 per cent of unchanged tosylate.

EXPERIMENTAL²

Oppenauer Oxidation of Methyl Hyodesoxycholate—5.54 gm. of methyl hyodesoxycholate³ (m.p. 108–110° after drying at 100° for 24 hours in a

²All melting points are corrected.

³Marker and Krueger (1) reported the melting point of this compound as 86° after crystallization from benzene. This product was undoubtedly a coordination compound with 1 mole of benzene, although correct analytical values were recorded. The air-dried product melts at 88–90° but is not clear up to 100°. If cooled slowly from 100°, the melt crystallized and on remelting softened at 120° and gave a clear melt at 123°. 48.7 mg. of the air-dried product after drying for 14 hours at 100° lost 7.1 mg.; calculated for C₂₅H₄₂O₄·C₆H₆ = 7.8 mg. The unsolvated ester then melted at 110–112°, $[\alpha]_D^{25} = +7.2^\circ$ (CHCl₃), and gave a correct analysis, C₂₅H₄₂O₄, calculated, C 73.84 H 10.41; found, C 73.66, H 10.51.

good vacuum) were dissolved in 50 ml. of anhydrous acetone and added to a solution of 6 gm. of aluminum *t*-butoxide in 200 ml. of dry benzene. The flask was stoppered and shaken at $40^{\circ} \pm 1^{\circ}$. After 51 hours 25 ml. of acetone and 75 ml. of benzene were added and shaking continued at the same temperature for a total of 119 hours. The solution was diluted with ether and washed thoroughly with dilute H_2SO_4 and with water. After removal of the solvent, the residue was dried at room temperature in a good vacuum, dissolved in 50 ml. of anhydrous pyridine, and 10 gm. of succinic anhydride were added. The solution was stored at 40° for 17 hours and then heated to 90° for 30 minutes. After cooling to 0° , water was added, and after a short interval the solution was diluted with ether and thoroughly extracted with dilute H_2SO_4 and with water. The ether solution was extracted with 1 per cent NaOH and the alkaline extracts were immediately acidified in a dilute H_2SO_4 -ice slurry. The ether solution was washed with water and the solvent removed. The residue weighed 1.68 gm. and is referred to as the "non-alcoholic fraction." The acidic fraction was extracted with ether, washed with water, esterified with diazomethane, and dried in a good vacuum at 45° . The product was separated into ketonic and non-ketonic fractions by means of the Girard Reagent T (12). The non-ketonic fraction which weighed 2.87 gm. is referred to as the "non-ketonic alcoholic fraction." The ketonic fraction was obtained by hydrolysis of the hydrazone with 0.2 N H_2SO_4 on standing overnight at room temperature and extraction with ether. This is referred to as the "ketonic alcoholic fraction."

The non-ketonic alcoholic fraction was hydrolyzed with 0.5 N NaOH for 30 minutes at room temperature and, after removal of a small neutral fraction by extraction with ether, the acid was obtained by acidification and extraction with ether. The acid was esterified with diazomethane and crystallized from benzene. 1.27 gm. of methyl hyodesoxycholate (m. p. $110-112^{\circ}$; no depression when admixed with an authentic sample) were obtained together with 0.69 gm. of amorphous material which could not be crystallized even after acetylation and chromatographic separation.

Methyl 3,6-Diketoallocholanate—The non-alcoholic fraction (1.68 gm.) was hydrolyzed at room temperature with 0.5 N NaOH. 67 mg. of neutral material were obtained by extraction with ether. The acid fraction was esterified with diazomethane and was purified by chromatographic separation on Al_2O_3 . 580 mg. of crystalline product were obtained which upon recrystallization from ethyl acetate-petroleum ether yielded 419 mg., m. p. $147-149^{\circ}$; $[\alpha]_D^{24} = +0.5^{\circ}$ ($CHCl_3$). The product gave no depression on admixture with an authentic sample of methyl 3,6-diketoallocholanate (m. p. $149.5-151^{\circ}$). No additional crystalline material was obtained upon further chromatographic separation.

3-Keto-6-hydroxycholanolic Acid—The ketonic-alcoholic fraction was hydrolyzed at room temperature for 30 minutes with 0.35 N aqueous alcoholic NaOH. 76 mg. of neutral material were extracted with ether. The acid fraction, weighing 1.77 gm., was crystallized from ethyl acetate and 965 mg., m.p. 172–177°, were obtained in two crops. After recrystallization once from ethyl acetate and three times from acetone the compound formed clusters of plates of constant melting point, 201–202°, with preliminary sintering at 198°; $[\alpha]_D^{22} = +13.7^\circ$ (acetone).

$C_{21}H_{33}O_4$. Calculated, C 73.81, H 9.81; found, C 73.56, H 10.03

Neither the methyl ester nor the methyl ester acetate of this compound could be obtained in crystalline state.

Methyl 3-Keto-6-p-toluenesulfoxycholanate—337 mg. of 3-keto-6-hydroxycholanolic acid were esterified with diazomethane, dried, and dissolved in pyridine. Approximately 2 gm. of freshly redistilled *p*-toluenesulfonyl chloride were added and the solution allowed to stand overnight. A small amount of water was added to the chilled solution and after a short time the solution was diluted with ethylene dichloride and with water. The aqueous portion was reextracted four times with fresh portions of ethylene dichloride; these were combined and washed with dilute acid, dilute base, and with water. After removal of the solvent the product was crystallized from ethyl acetate and three crops, weighing 300 mg. and melting at 173–183° with decomposition, were obtained. Recrystallization from ethyl acetate yielded rectangular prisms, m.p. 189–190° (with decomposition); $[\alpha]_D^{21} = +8.9^\circ$ ($CHCl_3$).

$C_{22}H_{34}O_6S$. Calculated, C 68.79, H 8.30, S 5.74; found, C 69.10, H 8.47, S 5.55

Methyl 3(α)-p-toluenesulfoxy-6-ketoallocholanate was prepared for comparison with the preceding product by a similar procedure. It crystallized from ether as long needles, m.p. 133.5–134.5°; $[\alpha]_D^{22} = -5.3^\circ$ ($CHCl_3$).

$C_{22}H_{34}O_6S$. Calculated, C 68.79, H 8.30, S 5.74; found, C 68.66, H 8.11, S 5.71

A mixture with methyl 3-keto-6-*p*-toluenesulfoxycholanate melted from 118°.

Methyl 3-Keto- Δ^4 -cholanate—275 mg. of methyl 3-keto-6-*p*-toluenesulfoxycholanate were heated under a reflux for 4 hours in 5 ml. of redistilled collidine. The solution was poured in ether and water, washed with dilute acid, dilute base, and with water, and after drying over Na_2SO_4 the solvent was removed. The slightly yellow crystalline residue weighed 204 mg. and was purified by chromatographic adsorption. After recrystallization from petroleum ether 150 mg. of prisms were obtained which melted at 124–125°; $\epsilon_{210} = 16,800$. Schoenheimer and Berliner (13) record a melting

point of 125° for the compound. Alkaline hydrolysis at room temperature yielded the free acid, m.p. 187°. Schoenheimer and Berliner (13) record a melting point of 185–186° for this compound.

317 mg. of methyl 3-keto-6-*p*-toluenesulfoxycholanate were heated under a reflux for 3 hours with 10 ml. of redistilled pyridine. The product was isolated as described above and yielded 168 mg. of unchanged tosylate together with 34 mg. of methyl 3-keto- Δ^4 -cholenate (melting point and molecular extinction coefficient).

223 mg. of methyl 3-keto-6-*p*-toluenesulfoxycholanate were dissolved in 5 ml. of acetonylacetone and heated under a reflux for 50 minutes with 234 mg. of sodium iodide. The solution was cooled and filtered, and the precipitate was washed with acetone and, after drying at 100° in a good vacuum, weighed 64 mg. The acetone was removed from the filtrate by distillation and the residue was dissolved in ether and washed thoroughly with water. There was considerable emulsification. The ether was removed and the residue was dissolved in 10 ml. of glacial acetic acid and heated under a reflux with 1 gm. of zinc dust for 2½ hours. The zinc was filtered off and washed with alcohol and the filtrate was evaporated to dryness. The residue after esterification with diazomethane was purified by chromatographic separation on Al_2O_3 . 98 mg. of a crystalline product, m.p. 109–111°, were obtained. This was hydrolyzed at room temperature with 0.5 *N* NaOH and the acid obtained was crystallized from ethyl acetate-petroleum ether. The product melted at 182–185° and had a typical ultraviolet absorption spectrum; $\epsilon_{260} = 19,000$. It did not depress the melting point of an authentic sample of 3-keto- Δ^4 -cholenic acid but when mixed with a sample of 3-ketoallocholanic acid (m.p. 182°) the melting point was depressed to 167°.

Preparation of 3-Keto-6-hydroxycholanolic Acid by Partial Saponification of Methyl Diacetoxhyhodesoxycholate—606 mg. of methyl 3,6-diacetoxycholanate (m.p. 99–101.5°) were dissolved in 20 ml. of methanol and 300 mg. of K_2CO_3 in 5 ml. of water were added. The ester crystallized but after 3 hours at room temperature with intermittent shaking redissolved. The solution was stored at room temperature (22–25°) for 24 hours and the methanol removed under diminished pressure at 25°. The solution was acidified under ether and extracted thoroughly. The ether solution was washed with water and the acid esterified with diazomethane. The resultant product was crystallized from benzene and 214 mg. of methyl hyodesoxycholate were obtained. The mother liquors on standing yielded an additional 20 mg. of the same compound. The amorphous residue weighed 276 mg. It was dissolved in 5 ml. of glacial acetic acid and 2.0 ml. of 3.3 *N* CrO_3 were added and the solution allowed to stand at room temperature for 5 hours. The excess CrO_3 was reduced with sodium bisulfite and

the reaction product isolated by extraction with ether. The ester was separated by chromatographing on Al_2O_3 . 35 mg. of methyl 3,6-diketoallocholanate were obtained which after recrystallization from petroleum ether melted at $146\text{--}148.5^\circ$; $[\alpha]_D^{22} = +2^\circ$ (CHCl_3). The compound gave no depression of the melting point on admixture with an authentic sample. The amorphous fraction from the chromatogram (86 mg.) after saponification at room temperature yielded 63 mg. of an acid melting at $193\text{--}197.5^\circ$. One recrystallization from acetone yielded plates, m.p. $202\text{--}203^\circ$, $[\alpha]_D^{23} = +14^\circ$ (acetone), which gave no depression on admixture with an authentic sample of 3-keto-6-hydroxycholanate acid.

SUMMARY

1. Methyl hyodesoxycholate was converted to methyl 3-keto-6-hydroxycholanate by Oppenauer oxidation at 40° .
2. Methyl 3-keto- Δ^4 -cholanate was prepared by heating methyl 3-keto-6-*p*-toluenesulfoxycholanate with collidine.

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THE EFFECT OF THE SYNTHETIC PTEROYLGLUTAMIC ACID ON THE FEATHERING OF CHICKENS

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The effect of cereal grains on feathering of chickens has been recently reviewed by Sanford and Wilcke (1). Hegsted *et al.* (2) showed the essential nature of certain amino acids to normal feather development. Mills *et al.* (3) demonstrated that the norit eluate factor of Hutchings *et al.* (4) promoted growth, feathering, and hemoglobin formation in chickens. Briggs *et al.* (5) believe that this fraction contains several factors, one of which is vitamin B₁₀, the feathering factor. Campbell *et al.* (6) have presented evidence of the feathering activity of vitamin B₆. Recently, Angier *et al.* (7) reported the synthesis of the *Lactobacillus casei* factor. The feathering activity of the synthetic pteroylglutamic acid forms the basis of the present report.

EXPERIMENTAL

Day-old white Leghorn chicks were housed in groups of ten in heated batteries. Food and water were fed *ad libitum*. Diets were prepared at weekly intervals.

The composition of Diet A is given in Table I. Diet B is identical except that *p*-aminobenzoic acid is omitted. Diet M is a commercial chick starter, fortified with 3 per cent each of dried liver cake, dried brewers' yeast, and Cerophyl.

The synthetic pteroylglutamic acid was dissolved in 0.05 N NaOH and added to the diets at the levels indicated. All other supplements were also added directly to the basal diets unless otherwise noted.

The method of scoring the feathers is similar to that used by the Wisconsin workers. A score of 100 represents perfect feathering. The normal range is from 80 to 100. A score of 60 to 80 indicates a fairly good feather growth, but the feathers are of somewhat inferior quality and are lacking in luster. A value of 50 or less indicates progressively poorer feather development. A very severe deficiency of the feathering factor is characterized by a score of 10 to 30.

Results

The results are summarized in Tables II to IV. The marked feathering effect of the pteroylglutamic acid is illustrated in Table II. It appears

that the amount necessary for maximum effect lies between 1.0 and 1.5 mg. of the compound per kilo of diet. Amounts up to 6 mg. per kilo have

TABLE I
Composition of Diet A

Ingredient		Ingredient	
	<i>per cent</i>		<i>mg. per cent</i>
Cerelose*	53.0	Niacinamide	3.0
Purified casein	22.0	Pyridoxine	0.5
Salts	4.3	Riboflavin	0.5
Calcium gluconate	3.0	Calcium pantothenate	3.0
Gelatin	8.0	Biotin	0.03
Ruffex†	4.0	Inositol	100.0
Soy bean oil	5.0	p-Aminobenzoic acid	5.0
Cholic acid	0.25	α-Tocopherol	5.0
Cystine	0.45	Menadione	0.2
	<i>mg. per cent</i>		<i>units</i>
Choline chloride	200.0	Vitamin A	3500
Thiamine "	0.3	" D	200

Orally once weekly, 7000 units of vitamin A, 400 units of vitamin D, 5 mg. of tocopherol, and 5 γ of menadione in corn oil.

* Glucose monohydrate.

† A purified cellulose containing 70 per cent α-cellulose and 30 per cent other celluloses (Fisher Scientific Company, Pittsburgh, Pennsylvania).

TABLE II
Effect of Pteroylglutamic Acid on Growth and Feathering in Chicks

Diet	Pteroylglutamic acid supplement per kilo	Average weight* at 28 days	Average feather score
	<i>mg.</i>	<i>gm.</i>	
A	None	91	24
"	0.5	161	34
"	1.0	236	49
"	1.5	261	71
"	2.0	269	73
"	2.5	242	68
"	5.0	226	68
M	None	289	95

* Values for the ten chicks alive in each group.

not further improved feather development. Pteroylglutamic acid-deficient chicks have a very poor growth of thin, brittle feathers. The addition of the synthetic compound produces very nearly normal feathering in most birds.

Since additional factors, as well as intestinal synthesis, are suggested to be involved in feather development (8), the experiments outlined in Tables III and IV were performed.

The addition of *p*-aminobenzoic acid to Diet B gives little or no effect above that of the basal diet plus pteroylglutamic acid. Thus, *p*-aminobenzoic acid does not seem to be necessary for feather development in the

TABLE III
Effect of Additional Supplements on Activity of Pteroylglutamic Acid

Diet	Supplement per kilo	Average weight and No. alive* at 25 days	Average feather score
		gm.	
B	None	98 (10)	26
"	3 mg. pteroylglutamic acid	247 (10)	67
"	3 " " " + 20 mg. <i>p</i> -aminobenzoic acid	277 (10)	74
"	3 mg. pteroylglutamic acid + 50 mg. <i>p</i> -aminobenzoic acid	305 (8)	73
"	3 mg. pteroylglutamic acid + 20 mg. <i>p</i> -aminophenylacetic acid	236 (10)	70
"	3 mg. pteroylglutamic acid + 200 mg. thyroid powder	248 (10)	72
M	None	295 (9)	93
A	1.5 mg. pteroylglutamic acid	266 (10)	61
"	6.0 " " "	249 (10)	69
"	6.0 " " " + 20 mg. β -pyracin	213 (10)	62
"	6.0 " " " + 20 " ascorbic acid (daily)	214 (10)	67
M	None	278 (10)	93
A	4.0 mg. pteroylglutamic acid	205 (9)	70
"	4.0 " " " + 5 gm. yeast nucleic acid	226 (10)	79
"	4.0 mg. pteroylglutamic acid + 25 gm. yeast nucleic acid	221 (10)	80
"	4.0 mg. pteroylglutamic acid + 100 gm. oats	269 (10)	82
M	None	265 (10)	88

* Figures in parentheses.

chick under the condition of our test. Additional experiments outlined in Table III demonstrate that little or no enhancement of the action of pteroylglutamic acid was obtained by addition to the diets of *p*-aminophenylacetic acid, thyroid powder, β -pyracin, ascorbic acid, yeast nucleic acid, pentanucleotides, or whole oats.

The effect of adding several intestinal antiseptics to Diet B is noted in Table IV. The addition of carboxysulfathiazole, sulfamerazine, *p,p'*-

diaminobenzophenone, or gentian violet to the basal diet did not appear to modify the effects obtained with pteroylglutamic acid. These results

TABLE IV
Effect of Intestinal Antiseptics on Activity of Pteroylglutamic Acid

Diet	Supplement per kilo	Average weight and No. alive* at 28 days	Average feather score
		gm.	
B	None	94 (7)	28
"	1.0 mg. pteroylglutamic acid	260 (8)	72
"	2.0 " " "	260 (10)	79
"	5.0 " " "	273 (7)	72
A	1.0 " " "	236 (7)	63
"	2.0 " " "	274 (8)	79
"	5.0 " " "	235 (10)	65
B	0.7% carboxysulfathiazole	114 (7)	10
"	0.7% " + 1.0 mg. pteroylglutamic acid	283 (7)	80
"	0.7% carboxysulfathiazole + 2.0 mg. pteroylglutamic acid	237 (7)	71
"	0.7% carboxysulfathiazole + 5.0 mg. pteroylglutannic acid	297 (10)	86
M	None	288 (6)	94
B	3.0 mg. pteroylglutamic acid	253 (10)	74
"	3.0 " " " + 0.5% sulfamerazine	188 (10)	65
"	3.0 mg. pteroylglutamic acid + 0.5% sulfamerazine + 200 mg. thyroid powder	223 (9)	78
"	3.0 mg. pteroylglutamic acid + 200 mg. <i>p,p'</i> -diaminobenzophenone	259 (10)	67
"	3.0 mg. pteroylglutamic acid + 0.2% gentian violet	269 (10)	60
M	None	281 (10)	92

* Figures in parenthesis.

TABLE V
Individual Variation on Mash and Synthetic Diets

Diet	Supplement per kilo	Individual scores	Average
M	None	90, 100, 90, 95, 95, 90, 80, 80, 90, 90	90
B	4 mg. pteroylglutamic acid	60, 25, 90, 90, 90, 90, 35, 80, 80, 70	71

would indicate that intestinal synthesis as a factor in normal feather development is of little or no significance.

DISCUSSION

It should be noted that while the data show that the average feathering on the synthetic diets containing pteroylglutamic acid is slightly subnormal, this is not entirely true. On the mash Diet M, all chicks show uniformly well developed feathers, while the birds receiving the synthetic diet show a definite variation, with one or two individuals having low scores and thus decreasing the average of a group containing a majority in the normal range. The individual scores of two typical groups are shown in Table V. The average differences are not statistically significant in groups of the size used.

From the data presented, pteroylglutamic acid can be considered as an essential dietary factor for the normal growth of feathers in chickens.

The effect of the pteroylglutamic acid is not lessened by the addition of certain intestinal antiseptics to the diet, thus indicating that intestinal synthesis of additional factors is not concerned in normal feather development.

Supplementing the diet with factors that have been suggested as playing a rôle in chick nutrition, i.e. *p*-aminobenzoic acid and ascorbic acid, does not increase the activity of the pteroylglutamic acid. The addition of thyroid powder, yeast nucleic acid, or whole oats to a diet containing pteroylglutamic acid did not significantly increase the quantity or quality of feathers produced.

SUMMARY

The synthetic pteroylglutamic acid is an essential dietary factor for the normal growth of feathers in chickens.

The effect of the pteroylglutamic acid is not enhanced by the addition of a number of biologically active compounds and its effect is not modified by the inclusion of certain intestinal antiseptics in the basal diet.

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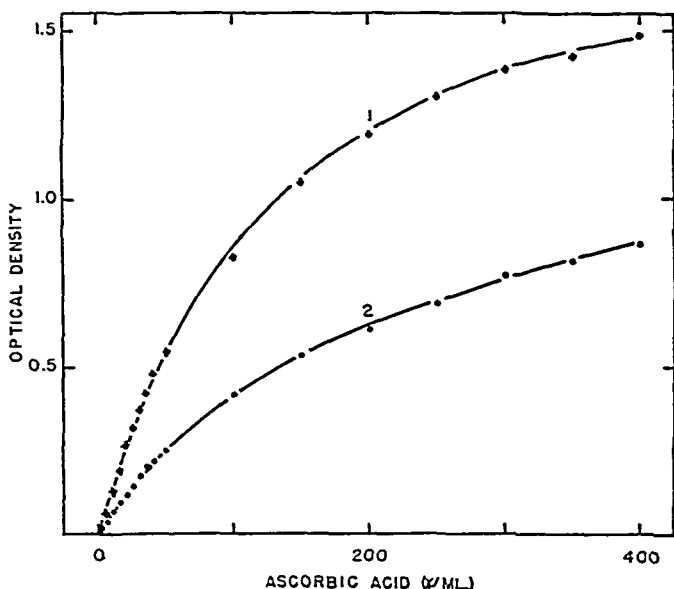
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LETTERS TO THE EDITORS

THE DETERMINATION OF ASCORBIC ACID; A SIMPLIFICATION OF THE ROE METHOD

Sirs:

To the present time no wholly satisfactory method has been developed for the determination of ascorbic acid.¹ The recent method of Roe *et al.*,² however, seems to offer better possibilities than do the more common methods in that it depends on the formation of a dinitrophenylhydrazone which has a characteristic color. Interfering ketones and aldehydes in



biological products appear to be of less consequence in the Roe method as modified by Penney and Zilva³ than do reducing substances in the 2,6-dichlorophenol indophenol methods.

¹ *Nutr. Rev.*, 2, 58 (1944).

² Roe, J. H., and Kuether, C. A., *J. Biol. Chem.*, 147, 399 (1943). Roe, J. H. and Oesterling, M. J., *J. Biol. Chem.*, 152, 511 (1944).

³ Penney, J. R., and Zilva, S. S., *Biochem. J.*, 39, 392 (1945).

A great deal would be gained in the Roe method by substituting for the 85 per cent sulfuric acid, employed to develop the color, a solvent that would not tend to heat and char the solution. Such a solvent would have the added advantages that less care would be required to dissolve the derivative and that much time could be saved in this step. Moreover, the method would be better adapted to mass routine analysis. Preliminary experiments for such a solvent indicated that glacial acetic acid could be employed to advantage.

The results plotted in the figure compare the optical density at 540 $m\mu$ obtained with 85 per cent sulfuric acid (Curve 1) and with glacial acetic acid (Curve 2). The fact that the reaction with acetic acid is only about half as sensitive as it is with 85 per cent sulfuric acid is of little importance.

Preliminary experiments on lettuce, cabbage, and citrus fruits indicate that the modification is a great improvement in the method, as the solutions do not char even on long standing. When sulfuric acid is employed on lettuce or cabbage, charring almost invariably occurs before the samples are ready to read in the colorimeter.

The acetic acid may be added in bulk to the dinitrophenylhydrazone suspension by means of a "volustat" without the necessity of cooling the reaction mixture in an ice bath. Thus, large scale, routine analysis may be greatly speeded up by substituting acetic acid for sulfuric acid in the Roe method for the determination of ascorbic acid.

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OXIDATION OF STEREOISOMERS OF THE INOSITOL GROUP BY ACETOBACTER SUBOXYDANS

Sirs:

The well known ability of *Acetobacter suboxydans* to dehydrogenate polyhydroxy compounds to ketones is, in the case of *meso*-inositol, illustrated by the production of a monoketoinositol.^{1,2}

Experiments on the oxidation by this microorganism of several stereoisomers of the inositol group have revealed interesting steric influences (see the table). Whereas *meso*-inositol, in accordance with previous

Oxidation of Inositols by *Acetobacter suboxydans*

The Warburg vessels in each experiment contained 0.5 cc. of the suspension of resting bacteria (about 20 mg., dry weight), 2 cc. of 1/15 M phosphate buffer of pH 6.0, and 0.5 cc. of substrate solution. The experiments were carried out at 38° in the presence of air.

Substrate		Oxygen consumption, moles per mole substrate	Duration of oxygen uptake
	micromoles		min.
<i>meso</i> -Inositol	6.6	0.47, 0.50	10
	13.9	0.50, 0.50	15
<i>l</i> -Inositol	6.6	1.01, 1.03, 1.00	20-25
<i>d</i> -Inositol	10.0	1.00, 0.97, 0.99	50
<i>epi</i> -Inositol	8.7	0.51, 0.49	10
<i>epi</i> -Inosose	6.8	0.25	15
	13.4	0.27, 0.27	15
	17.7	0.25, 0.26	20
Quebrachitol	12.5	0	
Pinitol	7.8	0	

findings,¹ consumed 0.5 mole of oxygen, the optically active isomers, *l*-inositol and *d*-inositol, required 1 mole. The naturally occurring monomethyl ethers of the *l* and *d* isomers, quebrachitol and pinitol, were not dehydrogenated.

It was found possible to demonstrate the formation of an α -diketo derivative of inositol in solutions of *l*-inositol exposed to the action of *Acetobacter* at 30° for 10 to 14 days by the isolation of a phenylosazone, crystallizing from aqueous pyridine or alcohol in long yellow needles with a greenish sheen and melting (with decomposition) at 213-214°. Found,

¹ Kluyver, A. J., and Boezaardt, A. G. J., *Rec. trav. chim. Pays-Bas*, 58, 956 (1939).

² Posternak, T., *Helv. chim. acta*, 24, 1045 (1941); 25, 746 (1942).

C 60.8, H 5.8, N 15.4; calculated for $C_{18}H_{20}O_4N_4$ (356.4), C 60.7, H 5.7, N 15.7. The vicinal position of the carbonyl groups was indicated by the consumption of 3 moles of periodic acid by the osazone. An osazone with similar properties was obtained, although in smaller yield, from *d*-inositol.

epi-Inosose³ (formed by the action of nitric acid on *meso*-inositol) and *epi*-inositol³ (obtained by the catalytic hydrogenation of *epi*-inosose) consumed 0.25 and 0.5 mole of oxygen respectively. The examination of culture fluids containing *epi*-inositol led to the isolation (via its phenylhydrazone) of a monoketo derivative of inositol, colorless needles which melted (with decomposition) at 198°, reduced Benedict's solution in the cold, and exhibited a slight levorotation, $[\alpha]_D^{27} = -5^\circ$. Found, C 40.4, H 5.7; calculated for $C_6H_{10}O_6$ (178.1), C 40.5, H 5.7.

When the specific steric requirements for the occurrence and the extent of oxidation are more clearly defined, this approach may become a useful tool in configurational studies on cyclitols. A detailed report on these experiments, which are being continued, will be presented later.

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³ Posternak, T., *Helv. chim. acta*, **19**, 1333 (1936)

DETECTION OF BIOCHEMICAL MUTANTS OF MICROORGANISMS

Sirs:

Biochemical or nutritional mutants of microorganisms have many uses in biochemistry and in chemical genetics,¹ but their application to many specific problems has been limited by the effort that must be spent to obtain the specific mutants required in a given instance. The methods that have been described previously² may be summarized briefly as follows:

Mutations are induced in a culture of the microorganism by any of a variety of agents, including x-radiation, ultraviolet light, and nitrogen mustard gas.³ Even with the most efficient mutating agents, only a small fraction of the cells in the culture are mutants. In order to detect the mutants, numerous single spore or single colony isolations are made, and cultures are established and maintained on a richly supplemented or "complete" medium. Each culture is then tested for its ability to grow on the minimal medium. The occasional strains which lack this ability are classed as mutants, and their specific nutritional requirements may then be determined. Since most of the cultures represent the original wild type, a great many tests must be made for the isolation of relatively few mutants, of which again, only certain ones may be of particular interest.

The following procedure has been developed to facilitate the detection of biochemical mutants. It depends on selecting the unmutated cells by their ability to form large colonies on a minimal agar medium, and the detection of the residue of mutants by the subsequent addition of a multiple supplement which will then allow the mutants to proliferate. The method has been applied primarily to the detection of mutants in ultraviolet-treated *Escherichia coli*, but there is no apparent reason why it should not be equally applicable to any organism which forms compact colonies when grown on minimal agar medium.

As applied to *Escherichia coli*, a minimal agar plate is poured in three layers, to the middle one of which 50 to 400 of the cells to be screened have been added while the agar was still liquid. The purpose of the stratification is to insure a uniform depth for all the colonies, to prevent spreading growth which is more liable to occur at a glass or air interface, and to keep the

¹ Beadle, G. W., *Chem. Rev.*, **37**, 15 (1945).

² Beadle, G. W., and Tatum, E. L., *Am. J. Bot.*, **32**, 678 (1945). Gray, C. H., and Tatum, E. L., *Proc. Nat. Acad. Sc.*, **30**, 404 (1944). Roepke, R. R., Libby, R. L., Small, H. H., *J. Bact.*, **48**, 401 (1944).

³ Tatum, E. L., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, in press (1946).

cells in a colony from contaminating the entire plate in the next step. The plates are incubated until the unmutated cells have developed into colonies of a convenient size (as experimentally determined). Then a layer of "complete" agar medium is poured on the surface of the plate. Growth factors diffusing through the agar reach the unproliferated mutant cells and permit their development into visible colonies. In *E. coli*, these colonies of mutant cells may be detected by their uniquely small size 6 to 24 hours after the "complete" medium has been added. The small colonies are picked to complete medium, and retested for their ability to grow on minimal medium, to verify whether they are indeed mutants. In the case of *E. coli*, most, but not all, of the small colonies have been demonstrably mutants, but there are indications that highly unstable mutants occur, which revert readily to the wild type.⁴ Such mutants would behave like the wild type subsequent to their initial isolation.

The method has been applied successfully to mixtures of wild type and previously isolated mutants. It has, furthermore, been used to isolate a variety of biochemical mutants from x-ray and ultraviolet-treated *E. coli*, including strains blocked at some point in the synthesis of proline, methionine, histidine, isoleucine, cystine, thiamine, or *p*-aminobenzoic acid.

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⁴ Ryan, F. J., and Lederberg, J., unpublished experiments.

⁵ Fellow of the Jane Coffin Childs Memorial Fund for Medical Research.

BIOTIN AND AVIDIN INTAKE AND LIVER CHOLESTEROL

Sirs:

Feeding rats biotin with diets very low in cholesterol has been shown by Gavin and McHenry¹ to give fatty livers containing 0.67 to 1.25 per cent cholesterol. When we fed adequate diets containing 1 per cent cholesterol,

*Lipid Content of Livers of Young Rats**

Diet†	Critical ingredients	No. of rats	Fatty acid‡	Total cholesterol‡	Notes
A	36% dried whole egg, no added biotin	8	5.0- 6.5	0.70-1.1	Livers seem normal; deficiency§ evident in males only
B	36% dried whole egg + biotin (1.5 γ per 10 gm. diet)				
	Sample I	4	7.2- 7.25	1.46-1.50	Livers fatty
	" II	4	3.0-12.0	2.31-2.33	" "
C	10.8% dried egg white, 1% cholesterol, no biotin				
	1 mo.	4	7.2- 8.4	0.90-0.93	Livers seem normal; beginning deficiency
	2 mos.	3	3.0- 3.2	0.20-0.22	Deficiency evident in all animals
D	10.8% dried egg white, 1% cholesterol, low biotin¶	8	3.2- 4.5	0.34-0.45	Deficiency§ evident in males only
E	Controls; 10.8% dried egg white, no cholesterol, low biotin¶	8	2.5- 6.0	0.26-0.50	Deficiency not obvious
F	Controls; casein, 1% cholesterol, vitamins as in Diet D	8	10.3-13.0	3.27-3.94	Livers fatty

* Equal numbers of males and females.

† The diets furnished, per kilo, 0.5 gm. each of choline and inositol and 2 mg. of folic acid. They were fed for at least 60 days unless otherwise noted.

‡ Per cent moist weight; ranges; livers from two rats pooled for each analysis.

§ First symptom, loss of hair.

|| Two Sample II contained less avidin than Sample I.

¶ 0.4 γ per 10 gm. of diet for 5 weeks, then 1.5 γ per 10 gm. for 7 weeks.

liver cholesterol values varied from about 3 to 8 per cent. We observed that when guinea pigs were given equal amounts of cholesterol (0.66 per

¹ Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, **141**, 619 (1941).

cent) in egg yolk, in whole egg,² or with casein, those fed the whole egg had about half the liver lipide found in the other groups. Feeding 2 γ of biotin per day with the casein-cholesterol diet doubled liver cholesterol values. This suggested that the avidin in the whole egg decreased storage of excess dietary cholesterol.

Further tests were made with rats. Diets adequate for growth were made up with vitamin-free casein, fat, salts, sucrose, and crystalline vitamins. Egg products, biotin, and cholesterol were incorporated in these diets as noted in the table. Cholesterol-fed rats with incipient biotin deficiency (Diets A, C, and D) stored no excess liver lipide, although they ate enough cholesterol-rich diet to gain weight normally up to the time of autopsy. Feeding small amounts of biotin (Diets B) or substitution of casein for egg white (Diet F) produced marked increases in liver cholesterol.

These data indicate that biotin may be essential for deposition of excess dietary cholesterol in the liver. Avidin feeding, presumably by making biotin unavailable, prevents cholesterol accumulation and there is even some indication that it may help to remove cholesterol already deposited. Cholesterol-fed rats given avidin for a short time have consistently more liver cholesterol than those fed for longer periods. Further work on this problem has been undertaken in cooperation with the division of Poultry Research.

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² The yolk of an egg is rich in biotin, but the white usually contains more than enough avidin to inactivate the yolk biotin.

THE OXIDATION OF OCTANOATE BY NORMAL AND LEUCEMIC MOUSE LIVER HOMOGENATES*

Sirs:

We have recently begun a study of enzyme systems in normal and leucemic mouse tissues, with particular emphasis on certain aspects of lipid metabolism.

As a first approach we have employed the technique of Lehninger¹ in studying fatty acid oxidation by liver homogenate systems. The mouse liver systems have been found to parallel rat liver in requiring adenosine triphosphate for octanoate oxidation. In order to obtain satisfactory Q_{O_2} values for mouse liver homogenates we have used one-half the substrate concentrations employed by Lehninger. In these experiments we have not

Average Oxygen Uptakes

45 minute period; approximately 85 mg. of fresh tissue per Warburg flask. The figures in parentheses represent the range.

	No. of animals	Complete system	Endogenous respiration (no substrate)	Net uptake
		<i>microliters</i>	<i>microliters</i>	<i>microliters</i>
Normal C-57 mice	7	164 (119-207)	82 (41-102)	82 (67-105)
Normal C-58 mice	3	205 (197-212)	109 (102-112)	96 (94-100)
Leucemic C-58 mice	7	37 (17-68)	32 (13-64)	5 (2.8-8.5)

used malonate to inhibit the so called endogenous respiration, and our systems have shown a definite cytochrome requirement.²

In the table are shown average oxygen uptakes for liver homogenate systems from seven normal mice of the C-57 Black strain,³ three non-leucemic animals of the MacDowell C-58 strain, and seven leucemic C-58 animals.

The data present a similar picture whether calculated on a wet or dry

* The authors wish to express their appreciation to Dr. E. C. MacDowell of the Department of Genetics, Carnegie Institution, Cold Spring Harbor, Long Island, New York, for supplying them with animals from the valuable C-58 strain, which shows a very high incidence of spontaneous leucemia.

¹ Lehninger, A. L., *J. Biol. Chem.*, 157, 363 (1945); 161, 437 (1945).

² Potter, V. R., *J. Biol. Chem.*, 163, 437 (1946).

³ Animals from the C-57 Black strain were kindly supplied by Dr. Herman B. Chase of the Zoology Department of the University of Illinois.

weight basis. Four of the leucemic animals showed marked fatty infiltration of the liver. A pathological study of the C-58 animals showed leucemic infiltrations in liver, lungs, kidneys, pancreas, and spleen and greatly enlarged lymph nodes in animals with leucemia. The non-leucemic C-58 and the C-57 mice presented a normal clinical and histological picture.

Studies of the anaerobic glycolysis of liver slices from the C-58 mice used in these experiments have provided some indication as to the extent of leucocyte infiltration in leucemic animals.⁴ Estimates were also made by histological means of the extent of leucemic infiltration. On the basis of such estimates malignant leucocytes occupied about one-sixth of the total tissue space of leucemic livers.

It appears from the results obtained that these leucemic liver systems are incapable of normal fatty acid oxidation. The evidence supports the idea that the presence of malignant leucocytes in the liver leads to failure of at least one important enzyme system. This work is being extended at present to include the study of other enzymes.

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⁴ Burk, D., Sprince, H., Spangler, J. M., Boon, M. C., and Furth, J., *J. Nat. Cancer Inst.*, **3**, 249 (1942).

THE GROWTH-STIMULATING EFFECT OF A HEAT-LABILE FACTOR IN LIVER EXTRACT ON RATS FED A NATURAL DIET

Sirs:

In studies on the nutritional value of certain human diets, evidence has been obtained for the existence of a heat-labile factor or factors in liver extract active to stimulate growth of rats. When certain natural diets were used, poor growth was obtained which could be stimulated by a liver fraction. Later it has been shown that the liver factor was heat-labile. The most striking growth stimulation was obtained with a diet of autoclaved soy beans and ground yellow corn, each fed *ad libitum*.

Growth Response of Rats Fed Autoclaved Soy Beans and Ground Yellow Corn to Liver Extract and Some Other Supplements

No. of animals	Supplement	Average weight gain in 9 wks.
24	None	69.5
20	Liver extract	131
8	Heated liver extract	71
4	Fresh yeast	112
8	Autoclaved yeast	103
4	Fresh yeast + liver extract	145.5
4	Casein + B vitamins	72.5

Liver extract and vitamins were fed 1 drop per animal per day, fresh yeast and casein in the amount of 0.5 gm. per animal per day, autoclaved and dried yeast 0.125 gm. per animal per day. The vitamin mixture contained in each ml. thiamine 1 mg., riboflavin 1 mg., pyridoxine 1 mg., pantothenic acid 2 mg., niacin 2 mg., choline 100 mg., inositol 25 mg., p-aminobenzoic acid 10 mg.

Rats maintained for 2 years on this diet showed fair growth, physical conditions, and reproduction. The liver preparation used to supplement this diet was prepared by alcohol extraction of fresh beef liver; proteins and lipides were removed from the extract. In the table the growth of various groups of rats fed the above diet plus a number of supplements is reported. It will be noted that the liver extract doubled the growth rate of the animals. If heated to 80° for 10 minutes, it was completely inactivated. Yeast, although stimulating growth, probably does not contain the active substance, as its action was not modified by autoclaving, and as liver stimulated growth of rats fed the diet plus fresh yeast. Casein plus a mixture of the known B vitamins (biotin and folic acid not included) was ineffective in stimulating growth. The active liver factor is probably

not identical with one of the known vitamins or amino acids, as they are not inactivated by short autoclaving in neutral solution and as they are present in yeast.

Woolley has lately described a factor, strepogenin, which stimulates growth of certain bacteria and mice; as it is heat-stable and present in casein,¹ it cannot be identical with the liver factor. While the present experiments have been under way and after their conclusion, a number of papers have been published reporting on a factor or factors in raw liver which stimulate growth of rats and mice,² cause optimal growth and prevent anemia in monkeys,³ and stimulate growth of *S. faecalis*.⁴ It remains to be shown whether all these actions are caused by the same factor.

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¹ Woolley, D. W., *J. Biol. Chem.*, **162**, 383 (1946).

² Jeminez-Diaz, C., and Vivanco, F., *Rev. clin. españ.*, **3**, 263 (1943). Bosshardt, D. K., Cierzko, L. S., Buffington, A. S., and Arnow, L. E., *Arch. Biochem.*, **7**, 1 (1945). McIntire, J. B., Henderson, L. M., Schweigert, B. S., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, **54**, 98 (1943).

³ Cooperman, J. M., Waisman, H. A., McCall, K. B., and Elvehjem, C. A., *J. Nutr.*, **30**, 45 (1945).

⁴ Cooperman, J. M., Ruegamer, W. R., Snell, E. E., and Elvehjem, C. A., *J. Biol. Chem.*, **163**, 769 (1946).

⁵ Present address, Department of Biochemistry, University of Wisconsin, Madison.

A SUBSTANCE IN INCUBATED RAT HEART AUGMENTING THE CHOLINESTERASE ACTIVITY OF HEART MUSCLE*

Sirs:

In the course of a study of the cholinesterase present in comminuted rat heart muscle, a peculiar property of this enzyme preparation¹ became apparent: Increasing activity towards acetylcholine² was observed, the increase reaching its maximum (30 to 60 per cent) after the preparation had been kept for 3 to 5 days at 2° or for 4 to 5 hours at 37°. Furthermore, when the 4 to 5 hour incubation period at 37° was followed by incubation at 28-30° for 16 hours, the tissue pulp was found to have acquired the ability to increase by 20 to 25 per cent the cholinesterase activity of an equal amount of fresh heart pulp.

The ability of a preparation, treated in the above manner, to increase the cholinesterase activity of fresh heart pulp was unaffected by subjecting the preparation to a temperature of 56° for 1 hour, a procedure which inactivates its cholinesterase. Heating the preparation to 56° before incubating it for 16 hours at 28-30°, however, prevented the release of the substance responsible for the activation of the cholinesterase in fresh heart pulp; this indicates that the release of the activating agent is due to an enzymatic process. The above results were also obtained under sterile conditions, the sterility being confirmed by bacteriological tests. The enzyme systems concerned with the formation and the release of the activating agent must, therefore, be present in the heart and were not introduced through contamination.

The activating effect of incubated heart pulp seems to be specific for the cholinesterase of heart, since no augmentation in the activity of cholinesterases from other sources could be demonstrated.

Dialysis or incineration of heart pulp previously shown to possess activating properties abolishes the ability of the preparation to activate the cholinesterase of fresh heart pulp.

The increase in activity of incubated heart preparations and the ability of these preparations to increase the cholinesterase activity of fresh heart pulp could result either from the formation of an activator or from the

* This investigation was aided by a grant from the Banting Research Foundation.

¹ A suspension of 1 part of finely ground rat heart in 3 parts of distilled water.

² The cholinesterase activity was measured manometrically at 37.5° in 0.025 M NaHCO₃ saturated with 5 per cent CO₂ in N₂. 0.15 cc. of the heart suspension was placed in the main compartment of the Warburg vessel and 0.05 cc. of acetylcholine (10 per cent) in the form of the chloride was tipped in from the side arm; the total fluid volume was 5.0 cc.

production of a substance which neutralizes the action of a naturally occurring cholinesterase inhibitor.

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THE INFLUENCE OF A STREPOGENIN CONCENTRATE ON THE METABOLISM OF GLUTAMIC ACID BY STREPTOCOCCUS FAECALIS*

Sirs:

The unusual growth response of *Lactobacillus arabinosus* toward limiting concentrations of glutamic acid in the medium was noted by Lewis and Olcott¹ and Lyman and coworkers.² We have attempted to use *Streptococcus faecalis* as the test organism in similar assays without success. Of particular interest was the fact that some unhydrolyzed crude materials resulted in dosage response curves at variance with the standard curves. Also, as with *Lactobacillus arabinosus*, amounts of glutamic acid adequate

Response of *Streptococcus faecalis* to Various Combinations of Glutamic Acid and Strepogenin

Glutamic acid per 10 cc. basal medium	Added strepogenin concentration per 10 cc. basal medium	Time required for growth to produce 0.16 mg. organisms	Generation time
mg.	mg.	hrs.	hrs.
0.1	0.2	26.0	
0.2	0.2	16.6	
0.3	0.2	13.0	
0.5	0.2	10.0	
0.2	1.0	7.5	
0.2	2.0	6.5	
0.2	0	47.4	6.2
0.4	0	23.4	2.5
0.7	0	14.5	1.6
1.0	0	11.3	1.2
2.0	0	7.9	0.87

for good growth sometimes required more than 100 hours before growth reached its limit, while larger amounts resulted in very rapid growth. Normally, growth is complete with this organism in 16 to 18 hours. These facts suggested to us that strepogenin³ might affect the early growth of *Streptococcus faecalis* at low glutamic acid concentrations. A concentrate of the growth factor was therefore prepared³ and tested.

* Research paper No. 589, Journal Series, University of Arkansas. Aided by a grant from the Committee on Therapeutic Research, Council on Pharmacy and Chemistry, American Medical Association.

¹ Lewis, J. C., and Olcott, H. S., *J. Biol. Chem.*, 157, 265 (1945).

² Lyman, C. M., Kuiken, K. A., Blotter, L., and Hale, F., *J. Biol. Chem.*, 157, 395 (1945).

³ Sprince, H., and Woolley, D. W., *J. Am. Chem. Soc.*, 67, 1734 (1945).

Media and methods similar to those of Baumgarten *et al.*⁴ were used. Growth was measured by reading turbidities with a spectrophotometer. As is shown in the table, the data obtained indicate that in the presence of added strepogenin the time required to produce a standard amount of growth is a function of the concentrations of both glutamic acid and strepogenin. When no strepogenin was added, the generation time was inversely related to the glutamic acid concentration. These data appear to establish a relationship between some substance in the strepogenin concentrate and glutamic acid metabolism. The concentrate showed similar activity when added before autoclaving or when sterilized by filtration and added after autoclaving the medium. Assay of the unhydrolyzed strepogenin concentrate for glutamic acid indicated that it had an activity corresponding to admixture with 5 to 10 per cent of its weight of glutamic acid.

Preliminary data obtained with *Lactobacillus casei* suggest that the same relationship holds with this organism. Therefore, there is little reason to doubt that the active factor is actually "strepogenin" as measured by the method of Sprince and Woolley.³

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Received for publication, August 8, 1946

⁴ Baumgarten, W., Garey, J. C., Olsen, M. J., Stone, L., and Boruff, C. S., *J. Am. Chem. Soc.*, **66**, 1607 (1944).

ACTION OF CHOLINE AND FAT ON THE FORMATION OF PHOSPHOLIPIDES IN THE INTESTINE*

Sirs:

Previous studies¹ have shown that lipid phosphorylation in the small intestine is more active when fat is ingested.² The present data indicate that both choline and fat are involved, a finding which may be of interest in relation to a possible rôle of lipid phosphorylation in the absorption of fat from the intestine.³

Male albino rats (100 to 110 gm.) were maintained for 7 days on a low fat, low choline diet.⁴ In each experiment four rats, A, B, C, and D, were injected intraperitoneally with 0.5 cc. of a solution of Na_2HPO_4 , containing radioactive phosphorus (P^{32}). Shortly before the injection the following

Total Radioactivity* and Specific Activity† of Lipide P in Small Intestine of Rats

Diet	Hrs. after P^{32}	Substance given			
		Water	Choline	Oil + choline	Oil + water
Choline-deficient	3	84 (38)	134 (48)	151 (47)	82 (38)
"	6	132 (53)	143 (70)	267 (90)	133 (59)
"	24	111 (42)	116 (48)	181 (61)	121 (48)
Stock†	6	117 (52)	145 (52)	233 (62)	187 (67)

* Expressed in relative radioactive units (r.r.u.), the total dose injected in the animal being considered equal to 10^4 r.r.u.

† The figures in parentheses indicate the specific activity (total radioactivity in r.r.u. per mg. of lipid P in the intestine of a 100 gm. rat).

‡ Rockland rat diet (complete), containing 5.5 per cent of fat.

were administered by stomach tube: Rat A, water (1 cc.); Rat B, choline hydrochloride (30 mg. in 1 cc. of water); Rat C, oil (2.2 cc.)⁵ and choline hydrochloride (30 mg. in 1 cc. of water); Rat D, oil (2.2 cc.) and water (1 cc.).

* Aided by a grant from the John and Mary R. Markle Foundation.

¹ Artom, C., Perrier, C., Santangelo, M., Sarzana, G., and Segrè, E., *Boll. Soc. ital. biol. sper.*, **12**, 708 (1937). Artom, C., Sarzana, G., and Segrè, E., *Arch. internat. physiol.*, **37**, 245 (1938). Fries, B. A., Ruben, S., Perlman, I., and Chaikoff, I. L., *J. Biol. Chem.*, **123**, 537 (1938).

² In these studies the diets probably contained adequate amounts of choline or choline precursors.

³ Sinclair, R. G., *J. Biol. Chem.*, **82**, 117 (1929); **115**, 211 (1936). Verzář, F., and Laszt, L., *Biochem. Z.*, **270**, 24, 35 (1934). Artom, C., and Peretti, G., *Arch. internat. physiol.*, **42**, 1 (1935).

⁴ Casein 5 parts, dextrin 42, sucrose 42, Crisco 2.5, cod liver oil 2.5, salt mixture 4, ruffex 2. A mixture of pure B vitamins was added to the daily ration of the rats.

⁵ A commercial preparation of partially hydrogenated cottonseed oil.

After 3, 6, and 24 hours the rats were killed by decapitation and the lipides extracted from the small intestine. On the lipide extracts the radioactivity and the phosphorus content were determined as described.⁶ Representative data of experiments from rats on the choline-deficient diet, and also from controls on an adequate stock diet, are recorded in the table.

It appears that when single large doses of choline and fat are given simultaneously there is a considerable increase in both the total radioactivity and the specific activity of the intestinal phospholipides. Choline alone also stimulates the phosphorylation of lipides in the intestine, but to a lesser extent than when choline and fat are ingested. In rats on the choline-deficient diet, the administration of oil alone does not affect the total radioactivity or the specific activity values. This finding, in conjunction with the results obtained from rats on the stock diet, suggests that the supply of choline (or choline precursors) may represent a limiting factor for the formation of phospholipides during the absorption of fat from the intestine.

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Received for publication, August 8, 1946

⁶ Artom, C., *J. Biol. Chem.*, **139**, 953 (1941).

THE ADENOSINETRIPHOSPHATASE ACTIVITY IN THE PRESENCE OF CREATINE

Sirs:

Ljubimova and Engelhardt¹ reported an indirect effect of creatine on the adenosinetriphosphatase activity in a dialyzed aqueous extract of muscle. The extracts were practically inactive when adenosine triphosphate (ATP) alone was added, but when creatine was also added, there was a formation of both true inorganic phosphate and phosphocreatine. They assumed that the reaction was connected with the process of transphosphorylation between ATP and creatine.

It was stated by Price and Cori² that this activation by creatine was not due to the formation of phosphocreatine, because on fractionation of the aqueous extracts with ammonium sulfate the formation of phosphocreatine disappeared, while the effect of creatine in enhancing adenosinetriphosphatase activity persisted.

When I repeated these experiments prior to final publication of extensive and seemingly well controlled experimental material, I could not confirm them. An aqueous extract of rat or rabbit muscle was first dialyzed and then precipitated three times at pH 7 and 33 per cent saturation with ammonium sulfate. The final precipitate was dissolved and dialyzed. Inorganic phosphate was formed from ATP, but there was no acceleration of this reaction by creatine in the absence of formation of phosphocreatine.

This cast doubt on the validity of other experimental findings previously reported,² particularly the claim that the adenosinetriphosphatase activity which remained after treatment of myosin with acetone was enhanced by creatine. This could not be confirmed either. When the myosin is not carefully purified, it contains the enzyme which transfers phosphate from ATP to creatine and this enzyme survives the acetone treatment. Unless true inorganic phosphate is being determined, the formation of phosphocreatine will simulate an acceleration of adenosinetriphosphatase activity by creatine. When the muscle is extracted with water prior to the extraction of myosin with salt solution, or when the myosin is reprecipitated and washed three times, some adenosinetriphosphatase activity remains after the acetone treatment, but creatine has no effect on it and there is no formation of phosphocreatine. In the course of this reinvestigation it was found that calcium can replace magnesium in the activation of the enzyme that transfers phosphate from ATP to creatine.

¹ Ljubimova, M. N., and Engelhardt, W. A., *Biokhimiya*, 4, 716 (1939).

² Price, W. H., and Cori, C. F., *J. Biol. Chem.*, 162, 393 (1946).

The question whether the adenosinetriphosphatase activity which remains after the acetone treatment is a true separation of the enzyme from myosin will require further work.

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Received for publication, August 13, 1946

A LABILE IRON POOL

Sirs:

When radioactive iron is injected intravenously, the rate at which it is incorporated into the erythrocytes as hemoglobin is such that it is necessary to consider that a pool of iron or a labile form of storage iron exists which should be distinguished from the available iron stores.

A 70 kilo man may possess a total circulating hemoglobin mass of 900 gm. It has been demonstrated that the average "life" of the erythrocyte is about 125 days.¹ Consequently a man of this size would break down $900/125 = 7.2$ gm. of hemoglobin each day, liberating $7.2 \times 3.39 = 24.4$ mg. of iron.² In order to maintain his hemoglobin level, the subject must use the same amount of iron per day for hemoglobin synthesis. If 5 mg. of Fe^{59} are injected intravenously, and if this were to merge with the 24.4 mg. of iron derived from hemoglobin breakdown, the proportion of the radioactive iron which would enter the red blood cells should be $(24.4 \times 100)/(24.4 + 5) = 83$ per cent daily. Actually much less than this is taken up by the red corpuscles.

It has been stated³ that in the dog the amounts of iron in the circulating hemoglobin and in the available stores represent respectively 57 and 15 to 20 per cent of the total body iron. If the figures are applied to man, a person who possesses 900 gm. of circulating hemoglobin should carry about 1000 mg. of iron in the available stores. If a small dose, say 5 mg., of intravenously administered radioactive iron were to merge with the available stores, then $(24.4 \times 100)/(24.4 + 5 + 1000) = 2.4$ per cent should appear in the circulation each day.

We have made estimates of the uptake of Fe^{59} with comparable doses in several species of animals and find values which rise from about 10 per cent on the 1st day to steady levels of about 15 per cent per day after the 2nd day. From the latter figure it can be calculated $((24.4 \times 100)/(24.4 + 5 + x) = 15)$ that the labile pool of iron (x) available amounts to about 133 mg. Calculated in relation to the body weight of the animal, the average figures for normal rats, pigs, and men, respectively, are 1.00, 1.68, and 3.69 mg. per kilo. These are based on the uptakes after 1 or 2 days of small amounts of radioactive iron injected intravenously (0.03 to 0.30, 0.01 to 0.02, and 0.064 mg. per kilo of body weight in the respective species).

¹ Hawkins, W. B., and Whipple, G. H., *Am. J. Physiol.*, **122**, 418 (1938). Shemin, D., and Rittenberg, D., *Federation Proc.*, **5**, 153 (1946).

² Drabkin, D. L., *Am. J. Med. Sc.*, **209**, 268 (1945).

³ Hahn, P. F., *Medicine*, **16**, 249 (1937).

Figures based on the uptakes 2 to 4 days after injection would be different, since the uptake then is usually more rapid than earlier. The estimates presented are admittedly only approximate; accurate values will require further study.

The labile pool would appear to be an intermediate stage which receives iron from hemoglobin breakdown or from oral absorption or iron that is injected parenterally. When a relatively small dose of iron is given intravenously to a normal mature animal, this can be incorporated into the pool. Most of the injected iron appears in the red blood cells in about 10 days. On the other hand, if a large dose of radioactive iron is given, the iron needs for hemoglobin formation and the capacity of the pool are exceeded, and a portion of the iron will enter the stores.

Very recently Ross,⁴ using a different approach, has also arrived at the conclusion that a "metabolic pool" of iron exists.

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⁴ Ross, J. F., *Proc. Am. Soc. Clin. Invest.*, 33 (1946).

THE TEMPERATURE COEFFICIENT FOR THE DENATURATION OF CHORIONIC AND MARE SERUM GONADOTROPINS IN AQUEOUS AND IN UREA SOLUTIONS

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The denaturation of chorionic gonadotropin in 40 per cent aqueous urea solution has been found exceptional with respect to both the heat of activation and entropy of activation, but similar to other protein denaturations when compared on the basis of free energy of activation (1). The low rate (2) of inactivation of mare serum gonadotropin in 40 per cent aqueous urea solution at 37.5°, along with the above observation, prompted a study of the temperature coefficient for the inactivation of the mare serum hormone in 40 per cent aqueous urea solution. In the case of the chorionic gonadotropin, the inactivation was independent of concentration but the rate was such that a first order reaction could only be assumed on the basis that the reaction product was a stable substance with less biologic activity. The question arose concerning the free energy of the activation in the denaturation of chorionic gonadotropin compared with results for the mare serum hormone on the basis of total inactivation. The temperature coefficient for the inactivation of the reaction product of chorionic gonadotropin in 40 per cent aqueous urea solution was therefore determined and the original experiments were repeated with a hormone prepared by an entirely different procedure and of a higher degree of purity. A chorionic gonadotropin of E. R. Squibb and Sons was used; it had been prepared by tannic acid precipitation with subsequent fractionation, and assayed 300 I.U. per mg. in contrast to 100 I.U. per mg. for our original hormone. Finally, it seemed desirable to study the temperature coefficient for the inactivation of the two hormones in aqueous solution.

EXPERIMENTAL

The experimental details of the denaturation procedures and of the biologic assay have been described (1, 2). The same hormone preparations were used with the addition of the chorionic gonadotropin furnished by Squibb, as described in the introduction. 120 rats were used for the bioassay in the experiments concerned with the mare serum hormone in urea solution. This does not include the data used from the earlier publica-

tion (forty additional rats). 100 rats were used in the heat inactivation experiments. The temperature coefficient for the inactivation of the reaction product of chorionic gonadotropin in urea solution is based on a bioassay in which 100 rats (not including twenty rats used in the earlier publication) were employed. In the duplication experiments with the Squibb hormone, 160 rats were employed. 280 rats were used in the heat inactivation experiments. Since the accuracy of a bioassay is proportional to the square root of the number of animals employed and the heat of inactivation varies with the logarithm of the ratio of reaction rates, it was felt that increasing the number of animals within reasonable limits in the bioassay would give little additional information.

The bioassays in the isolated experiments, concerned with the inactivation of the chorionic gonadotropin in aqueous solution in the temperature range above 65°, were performed with the same degree of accuracy as in the other experiments described in this paper. The results are not incorporated in tabular form but reported in the body of the text. The statistic accompanying each result is the standard deviation of the mean.

Results

Mare Serum Hormone in 40 Per Cent Urea—The inactivation of pregnant mare serum gonadotropin by exposure to 40 per cent aqueous urea solution was studied at three temperatures; viz., 58.0°, 50.0°, and 37.5°. The recovery of hormone and reaction rates calculated from the recovery data are given in Table I. The agreement of reaction rates with time is well within the accuracy of the bioassay and establishes a first order reaction.¹ In Fig. 1, the logarithms of the reaction rates are plotted against the reciprocals of the absolute temperatures² and again the agreement is well within the accuracy of the bioassay.

If the velocity constants (taken from the graph, Fig. 1) 0.089 and 0.00105 for 58.0° and 37.5° respectively are substituted in the integrated form of the Arrhenius equation,³ Q , the activation energy for the reaction, becomes 44,100 calories mole⁻¹. The experimental error may be estimated by rotating the line in Fig. 1 by a distance, at each extreme temperature, equal to the standard deviation of the mean of the distances of the respective points to the line. On recalculating Q , the difference from the original

¹ $k = 1/t \times 2.3 \log C/(C - a)$, where t is the reaction time in minutes, C is the original concentration of hormone, and a is the amount of C inactivated in time t .

² $\log k = C - a/T$, where k is the velocity constant, T is the absolute temperature, and C and a are constants. If this relation holds, the logarithm of the velocity constant plotted against $1/T$ should result in a straight line.

³ $Q = R2.3 \frac{(\log k_2 - \log k_1)}{(1/T_1 - 1/T_2)}$, in which k_1 and k_2 are the rates at the absolute temperatures T_1 and T_2 , R is the gas constant in calories, and Q is the activation energy for the reaction in calories per mole.

TABLE I

Inactivation of Gonadotropin of Pregnant Mare Serum by Exposure to 40 Per Cent Aqueous Urea Solution at Various Temperatures

Temperature of inactivation	Time of inactivation	Ratio of control to experimental dose	Mean organ weight in bioassay		Estimated recovery of hormone	Correct assay range, 19 of 20 trials	k (min. ⁻¹)
			Control	Experimental			
°C.	min.		mg.	mg.	per cent	per cent	
58.0	5	1.0	91 ± 11 (O.)	47 ± 4 (O.)	70	10	0.072*
	15	0.5	91 ± 11 "	53 ± 7 "	34	6	0.080†
	120	1.0	34 ± 3 "	14 ± 0.5 "			
50.0				16 ± 0.5 (U.)	7	Assured	
	5	1.0		40 ± 5 (O.)			
	30	0.84		27 ± 2.5 "	60	9	0.020
	60	0.5	55 ± 6 (O.)	35 ± 4 "	37	7	0.017
	120	1.0		13 ± 0.6 "			0.018‡
37.5				96 ± 6 (U.)	12	2	0.018§
	360				72		0.00091
	1080	0.5	50 ± 6 (O.)	29 ± 2 (O.)	35	4	0.00097
	1350				24		0.00105

O. indicates that the assay was performed by measuring the ovarian weight; U. by measuring the uterine weight.

* By comparing the inactivation at 15 and 5 minutes (litter mates).

† By correcting for the temperature lag from 0 time (litter mates).

‡ By comparing the inactivation at 5 and 120 minutes (litter mates).

§ By comparing the inactivation at 30 and 120 minutes (litter mates).

|| Data from previous publication.

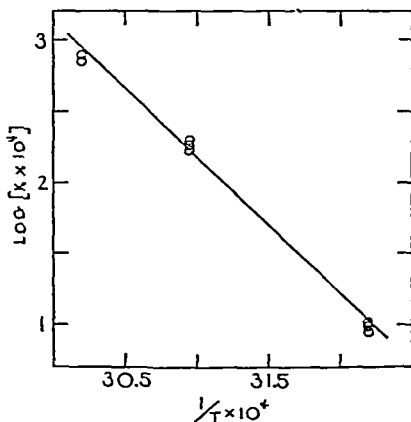


FIG. 1. Heat inactivation of mare serum gonadotropin in 40 per cent aqueous urea solution, pH 7.0. The log rate of inactivation is plotted against the reciprocal of the absolute temperature.

value is about 1000 calories, which may be regarded as once the standard deviation of the mean.

The entropy of activation may be calculated by use of the equation for the absolute rate of denaturation⁴ at 0°. The value of k , at 0°, may be obtained by the use of the Arrhenius equation, 44,100 calories being substituted for Q , and 0.00105 for k_2 , the rate at 310.6° (absolute temperature). The rate at 0°, which must be converted to seconds for use in the subsequent substitution, becomes $9.45 \times 10^{-10} \text{ sec.}^{-1}$. With this constant, the entropy of activation ΔS , which entails the denaturation, becomes 61

TABLE II
Inactivation of Gonadotropin of Pregnant Mare Serum in Aqueous 1 Per Cent NaCl Solution at pH 7.0 by Heat

Temperature of inactivation	Time of inactivation	Ratio of control to experimental dose	Ovarian weight in bioassay, mean \pm standard deviation of mean	Estimated recovery of hormone	Limit of per cent deviation, 19 of 20 times	k (min. ⁻¹)
°C.	min.		mg.	per cent	per cent	
80.0	5		43 \pm 5			
	20	0.8	32 \pm 2	68	9	0.026
	35	0.5	28 \pm 1.4	38	4	0.032
75.7	5		60 \pm 6	54		
	35	1.0	36 \pm 5	75	13	0.0096
75.1	5		54 \pm 5			
	80	0.72	29 \pm 1	49	5	0.0095
70.7	5		140 \pm 5			
	100	1.0	101 \pm 6	78	7	0.0026
67.0	0		114 \pm 8			
	360	1.0	55 \pm 7	63	7	0.0013
60.0	1440					0.00025*
	2880					0.00024*
	4320					0.00034*
	5760					0.00032*

* Data of Rimington and Rowlands.

calories degree⁻¹ mole⁻¹ and the free energy of activation⁵ becomes 25,200 calories mole⁻¹ at 37°.

Mare Serum Hormone in Aqueous Solution—The inactivation was performed at the temperatures given in Table II. The recovery of hormone

⁴ $k_0 = 5.7 \times 10^{12} \times e^{-Q/RT} \times e^{\Delta S/R}$, where Q , R , T , e , and ΔS have their usual meaning and 5.7×10^{12} is the ratio of Boltzmann's constant (1.346×10^{-16} erg) and Planck's constant (6.554×10^{-27} erg per second) times 273.1 (the absolute temperature at 0°).

⁵ $\Delta F = \Delta H - T\Delta S$, where ΔH is the heat of activation, ΔF is the change in free energy, and ΔS is the change in entropy.

and reaction rates calculated from the recovery are also given in Table II. Rimington and Rowlands have reported the rate at 60° and concluded that it follows an exponential course (3). This is confirmed by our data at 80.0°. In Fig. 2, the logarithms of the reaction rates are plotted against the reciprocals of the absolute temperatures. The data of Rimington and Rowlands are included and extrapolation of the plotted graph of our data to the temperature range used by Rimington and Rowlands shows excellent agreement with theory.

When the velocity constants 0.026 and 0.00024 for 80.0° and 60.0°, respectively, are substituted in the Arrhenius equation, Q , the activation energy for the reaction, becomes 51,700 calories mole⁻¹ with a standard

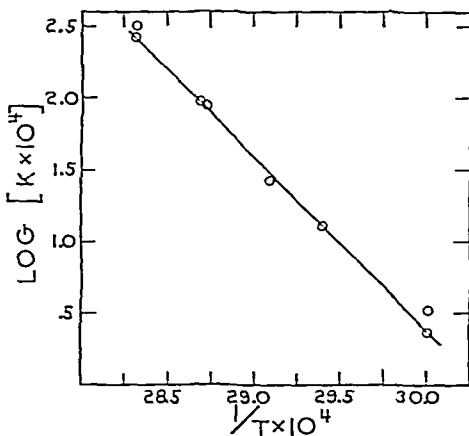


FIG. 2. Heat inactivation of mare serum gonadotropin in 1 per cent aqueous NaCl solution, pH 7.0. The log rate of inactivation is plotted against the reciprocal of the absolute temperature.

error of 1300 calories mole⁻¹. The reaction rate at 0° is calculated as 5.15×10^{-14} sec.⁻¹, the entropy of activation as 80.3 calories degree⁻¹ mole⁻¹, and the change of free energy at 37° as 29,800 calories mole⁻¹.

Chorionic Gonadotropin in 40 Per Cent Urea Solution—The slow reaction of chorionic gonadotropin in 40 per cent aqueous urea solution was studied after the fast reaction had become unmeasurable (6 hours). Three temperatures, 50.0°, 37.5°, and 22.5°, were selected and the reaction product was compared with the control hormone in 2.2 per cent (after dilution from 40 per cent urea concentration) urea solution incubated at the same temperature range. The results are given in Table III. The agreement of reaction rates with time is well within the accuracy of the bioassay

and establishes a first order of reaction; the change of reaction rate with temperature follows the Arrhenius law.

If the velocity constants (taken from a graph of $\log k$ plotted against $1/T$) 0.0027 and 0.00015 for 50.0° and 22.5°, respectively, are substituted in the Arrhenius equation,³ Q , the activation energy, becomes 20,000 calories mole⁻¹. The experimental error of this value, obtained in a manner analogous to that described in the mare serum hormone experiments, becomes 400 calories for once the standard deviation of the mean. According to the general scheme of calculation used for the mare serum hormone, the speed of reaction at 0° becomes 1.51×10^{-7} sec.⁻¹. The entropy of activation becomes -17 calories degree⁻¹ mole⁻¹ and the free energy of activation becomes 25,300 calories mole⁻¹ at 37°.

TABLE III

Inactivation of Denatured Chorionic Gonadotropin (Formed by 6 Hour Reaction in 40 Per Cent Urea Solution at 37.5°) in 40 Per Cent Urea Solution

Temperature of inactivation	Time of inactivation	Ratio of control to experimental dose	Mean organ weight		Estimated recovery of hormone	Correct assay range, 19 of 20 trials	k (min. ⁻¹)
			Control	Experimental			
°C.	min.		mg.	mg.	per cent	per cent	
50.0	236	0.50	39 ± 7	41 ± 6	52	11	0.0028
	465	0.33	48 ± 8	25 ± 1.3	20	3	0.0034
37.5	1080				48		0.00070*
	1080	0.50	99 ± 5	107 ± 4	53	11	0.00052
22.5	1440	0.50	40 ± 8	25 ± 3	32	8	0.00079
	3940	0.75	80 ± 3	50 ± 8	53	10	0.00016
	6835	0.50	75 ± 4	54 ± 5	39	5	0.00014

* Previous data.

Comparison of Squibb and Santa Barbara Chorionic Gonadotropins—The inactivation of the Squibb and the Santa Barbara chorionic gonadotropins in 40 per cent urea solution is compared in Table IV. Up to 120 minutes, the agreement is within twice the standard error. From this period to 1080 minutes, which represents the end of the rapid inactivation of the first product and the slow rate characterized by the second product, the Squibb chorionic gonadotropin reveals a lower value in all but one experiment, in which the value exceeds twice the standard error in two instances. The difference is, however, only between 1 and 2 per cent of the original activity of the hormone. Since chorionic gonadotropin slowly loses activity in aqueous solution (about 50 per cent in 1 month at room temperature), this slight difference probably represents a difference in the two preparations introduced at the source. Within the

slight difference the two preparations show remarkable agreement. The 1 per cent difference is also reflected in the inactivation by heat at 99°.

Since it is highly improbable that two preparations, as those described above, varying so widely in purity and method of preparation, should contain the same ratio of another hormone as an impurity, our original explanation that the reaction product is of lesser biologic activity is more tenable than assuming the presence of a preformed slowly reacting gonadotropin. The latter probability appears to have been eliminated.

Chorionic Gonadotropin in Aqueous Solution—It will be noted (Experiments 1 and 2, Table V) that at 65° chorionic gonadotropin is rapidly inactivated, 50 per cent of the activity being destroyed in less than 5 min-

TABLE IV

Comparison of Inactivation of Squibb and Santa Barbara Chorionic Gonadotropins in 40 Per Cent Urea Solution at 37.5°

Time of inactivation	Estimated recovery of hormone	Correct assay range, 19 of 20 times	Recovery previously obtained with Santa Barbara prolan
min.	per cent	per cent	per cent
None*	106	± 22	
30	39	± 5	38
60	24	± 5	21
120	15	± 3	12
124	11.7	± 2.8	12
180	6.4	± 1.5	(9.0 \pm 0.9)†
300	5.7	± 1.8	(8.1 \pm 0.9)
360	7.5	± 1.2	8.0 \pm 0.9
510	5.7	± 2.2	(6.9 \pm 0.9)
1080	4.4	± 0.6	3.8 \pm 0.7

* Control for effect of 6 per cent urea concentration in assay.

† Figures in parentheses, calculated from reaction rate.

utes. (In the experiments in Table V, 1.5 to 2.0 minutes were required for the reaction temperature to reach equilibrium.) However, at 15 minutes 24 per cent of the activity survives and 5 minutes additional heating produce no further inactivation. It will also be noted from the data of Experiments 7, 8, and 9 that increasing the concentration 4-fold has no significant effect upon the degree of inactivation. These results are interpreted as showing that the inactivation is of the first order, but that the reaction product possesses 24 per cent of the original activity. The concentration of original hormone in the course of the reaction would, therefore, be the apparent concentration minus 24/76 of the apparent inactivation. With this correction the velocity constants as given in Table V show good agreement; in Experiment 6, the time is doubled and in Experi-

ment 9, data, at four time intervals, the extremes of which are more than 5-fold, are well within the error of the bioassay.

The results obtained by plotting the logarithms of the velocity constants against the reciprocals of the absolute temperatures indicate a straight line function. Individual values deviate more from the theoretical than they do in the other experiments described in this paper; in the determination of the reaction rate for chorionic gonadotropin, the experimental

TABLE V
Inactivation of Chorionic Gonadotropin in 1 Per Cent Aqueous NaCl Solution, pH 6.3, by Heat

Experiment No.	Temperature of reaction	Time of reaction	Ratio of control to experimental dose	Mean uterine weight		Estimated recovery of hormone	Correct assay range, 19 of 20 trials	k (min. ⁻¹)
				Control dose	Experimental dose			
	°C.	min.		mg.	mg.	per cent	per cent	
1	65.0	5	0.5	83 ± 7	81 ± 7	50	10	
		15	0.2	83 ± 7	98 ± 4	24	5	
2	65.0	5	0.5	68 ± 10	67 ± 10	53	15	
		20	0.2	68 ± 10	79 ± 7	24	4	
3	62.5	5	0.5		33 ± 4	65	10	
		15	0.13		86 ± 8	35	6	0.14
4	60.7	15	1.0	46 ± 5	27 ± 3	66	14	0.046
5	59.0	30	0.5	89 ± 5	71 ± 8	41	8	0.054
6	58.0	60	0.5	43 ± 5	40 ± 5	45	7	0.023
		120	0.5	45 ± 5	24 ± 3	30	4	0.022
7	56.9	60	1.0	54 ± 7	26 ± 5	56	15	0.015
		60	1.0	61 ± 7	37 ± 6	63*	16	0.012
8	50.3	380	0.5	50 ± 6	61 ± 5	60	9	0.0020
		380	0.5	50 ± 6	64 ± 7	63*	7	0.0018
9	50.0	270	1.0	54 ± 7	29 ± 3	61	13	0.0026
		470	0.5	77 ± 6	80 ± 7	53	9	0.0021
		840	0.5	51 ± 7	29 ± 4	33*	7	0.0025
		1550	0.25	71 ± 5	85 ± 9	28	4	0.0019

* In these experiments the concentration was 400 i.u. per cc. instead of 100 as in all other experiments.

error is greater, because the calculation is based upon two bioassays instead of one. Although the number of rats used in the bioassay was doubled, the standard error of the Arrhenius constant is approximately 6 per cent as compared with 2 to 3 per cent for the three other Arrhenius constants determined.

The activation energy is calculated for the two velocity constants, 0.105 and 0.00195 at 62.5° and 50.0° respectively. *Q* becomes 68,800 calories mole⁻¹ with a standard error of 4000 calories mole⁻¹. The rate

at 0° becomes 9.42×10^{-14} sec.⁻¹. The entropy of activation is calculated as 133.4 calories degree⁻¹ mole⁻¹ and the free energy of the activation as 27,400 calories mole⁻¹ at 37°.

Chorionic Gonadotropin in Aqueous Solution above 65°—The rapid inactivation of chorionic gonadotropin in aqueous solution at 99–100° has been reported in earlier publications (4, 5). In less than 2 minutes, 95 per cent of the biologic activity is destroyed at this temperature. In exploratory experiments it was found that further inactivation proceeds slowly. Assuming the inactivation is a first order reaction, k (min.⁻¹) equals approximately 3×10^{-2} at 99°, a rate which is attained by the initial inactivation of the original hormone by heat in aqueous solution at a temperature approximately 40° lower. The recovery at 99° for a

TABLE VI

Comparison of Uterine and Ovarian Response of Immature Rats to Reaction Product of Chorionic Gonadotropin Obtained by Heat Treatment in Aqueous Solution at 99° for 10 Minutes

Substance injected	Dose per rat equivalent to original solution	Mean ovarian weight \pm standard deviation of mean	Mean uterine weight \pm standard deviation of mean	Per cent recovery of hormone	Per cent deviation 19 of 20 times
	cc.	mg	mg.		
Control gonadotropin, 400 i.u. per cc....	0.04	$23 \pm 0.8^*$		106	17
Same, heated 10 min. at 99°	0.125	12 ± 0.8	71 ± 7	4.4	0.6
" " 10 " " 99°	1.0	$25 \pm 1.0^*$		5.0	0.8

* Ovaries heavily luteinized.

reaction time of 40 minutes was 1.6 ± 0.15 per cent; at 10 minutes the recovery was 4.0 ± 0.5 per cent.

Below 65°, the heat inactivation of chorionic gonadotropin produced a reaction product of relative stability with 24 per cent of the original biologic activity. Essentially the same activity, 23 ± 2 per cent, was obtained in less than 5.0 minutes at 71.5°. 10 minutes additional treatment at this temperature resulted in an additional 50 per cent (of the 23 per cent) loss in biologic activity, the resulting product assaying 11 ± 1 per cent of the original activity. At 77.1°, 90 per cent of the original activity is destroyed in less than 5 minutes. In the next 5 minutes the activity fell from 10 ± 0.8 to 7 ± 0.8 per cent.

The results of the experiments in Table V and the exploratory work described above indicate that in the heat inactivation of chorionic gonadotropin at least two intermediary compounds are formed, one with 24 and one with 5 per cent of the original activity. There is probably a third

with approximately 10 per cent of the original activity. No attempt was made to study the temperature coefficients of these intermediary reaction products. It is apparent that the further inactivation of the first reaction product would have a high heat of activation.

Qualitative and Quantitative Response of Denatured Product—The ratio of the dosage levels required to produce varying responses in the uterine and ovarian weights of test animals is quite characteristic for the various gonadotropins, especially so for the chorionic gonadotropin. For the dosage level of either of the three hormones (chorionic, mare serum, and sheep pituitary) required to produce a uterine weight of 70 mg. in immature rats, 8 times this level are required to raise the ovarian weight to 24 mg. in the case of the chorionic gonadotropin, 4 times the level in the case of sheep pituitary gonadotropin, and only $2\frac{1}{2}$ times the level in the case of the mare serum hormone.

It seemed desirable to ascertain whether the reaction products of denatured chorionic gonadotropin retained this characteristic. The extreme case, in which heat denaturation at 99° in aqueous solution produces a reaction product with 5 per cent of the original activity (as assayed by uterine weight increases), was tested. The results are given in Table VI. Per cent recovery is calculated by use of the reference assay curves established for the hormone and an assay for the control is run simultaneously upon litter mates to show that the response of the rats fits the assay curve.

The results of this experiment show quite conclusively that, although the product retains only 5 per cent of the original activity, the effects produced upon ovarian and uterine weight are identical both qualitatively and quantitatively.

DISCUSSION

It is apparent from a summation of the results in Table VII that the inactivation of pregnant mare serum hormone, whether produced in 40 per cent urea solution or in 1 per cent aqueous NaCl solution at a higher temperature range, has the characteristics of a classic protein denaturation. The heats of activation and entropies of activation for the two reactions are of the same order, indicating a similar mechanism. These results are to be contrasted with those obtained for the chorionic gonadotropin, in which the evidence, while indicating first order kinetics for all the reactions studied, points conclusively to the formation of intermediate compounds. The low heats of activation and entropies of activation for the first and second reactions of chorionic gonadotropin in urea are more characteristic of normal reactions than reactions regarded as denaturations. The formation of an intermediate compound should not eliminate the possibility of a denaturation process, since a denaturation is probably a

summation of reactions, the measurement of rate depending on the slowest of these reactions. There is another example of this in the present studies, the inactivation of chorionic gonadotropin in aqueous solution by heat. In this case the high heats of activation and of entropy in the formation of a product with 24 per cent of the original activity are characteristic for those reactions which are considered denaturations. The difficulty of reconciling the two steps in the inactivation of chorionic gonadotropin by urea with a denaturation is that the sum of the free energy changes of both reactions, which is a measure for the over-all inactivation, is double the value regarded characteristic for a denaturation. The complete inactivation of chorionic gonadotropin in aqueous solution would also result in a great change in free energy. The heat denaturation of β -

TABLE VII

Comparison of Q , Activation Energy, ΔF , Change of Free Energy, and ΔS , Entropy of Activation, for Inactivation of Chorionic and Mare Serum Gonadotropins in 40 Per Cent Aqueous Urea Solution or in 1 Per Cent NaCl Solution by Heat

Reaction conditions for hormone	Q <small>calories mole⁻¹</small>	ΔF at 37° <small>calories mole⁻¹</small>	ΔS <small>calories degree⁻¹ mole⁻¹</small>
Mare serum in 40% urea, aqueous	44,100	25,200	+61
" " " 1% NaCl, "	54,700	29,800	+80
Chorionic, in 40% urea to product with 8% of activity	27,100	22,800	+14
Chorionic, 8% reaction product (above) in 40% urea	20,000	25,300	-17
Chorionic, in 1% NaCl to product with 24% of activity	68,800	27,400	+133

lactoglobulin has recently been shown by Briggs and Hull (6) to occur in two steps, the heat of activation of the first step being 48,100 calories mole⁻¹, that of the second step being 28,000 calories mole⁻¹. It is possible that further studies will yield more data of this nature, so that our results with the chorionic gonadotropin are not as unusual as was at first suspected.

One of the interesting results of our studies is the establishment of the existence of intermediary products of denaturation possessing biologic activity. These were first described in an earlier report (2) in which it was postulated that the biologic activity is dependent upon the spacing of active adsorption foci on the surface of the molecule, the spacing becoming deranged in dissociation of the molecule in denaturation. While producing the same biologic response, both qualitatively and quantitatively, these derivatives well might produce different antihormone or antigenic responses and should be investigated.

While the respective heats of activation for the mare serum hormone in water and aqueous urea are of the same order, the difference of 10,000 calories, which is 6 times the standard error, is highly significant. Assuming the mechanism of denaturation in the two environments are the same, the difference of reaction rates at the same temperatures should be related to the respective activity coefficients in water and urea solution (7). The heats of activation should, however, be the same, which is not the case. The difference of 10,000 calories more probably reflects a difference in the heats of solution of the hormone in water and aqueous urea. Since the heat of solution is a summation of the heats of sublimation and heats of solvation and since the heat of sublimation would be the same, the difference must be relegated to the heats of solvation; in the process of initial solution the energy required for solvation is greater in aqueous urea than in water and the resulting solute in urea may be regarded as an initial step in the total inactivation of the hormone. At this step there is no change in the biologic activity, as numerous assays have shown. Either the reaction is reversible or the spacing of secondary valence foci necessary for biologic activity has not been disrupted by the initial change in orientation. In the case of the chorionic gonadotropin the heats of activation reflect an even greater divergence, so that there would be an even greater change in the solute on initial solvation. Since the exact relation between heat of reaction and heat of activation is somewhat obscure and since the heat of solvation is a heat of reaction, the 10,000 calories cannot be regarded as an exact measure of difference in heat of solvation. The magnitude is, however, quite characteristic for a heat of activation of a normal reaction with a low temperature coefficient.

SUMMARY

The inactivation rates of pregnant mare serum gonadotropin in 40 per cent aqueous urea solution in the temperature range 37.5–50.0° and in aqueous 1 per cent NaCl solution in the temperature range 67–80° were studied with the following results.

The degree of inactivation was independent of concentration and the velocity constants calculated for a monomolecular reaction agreed within the accuracy of the bioassay, indicating simple first order kinetics with a reaction product devoid of biologic activity. The heats of activation, entropies of activation, and change of free energy at 37°, as calculated from the reaction rates, were characteristic for a protein denaturation. For the inactivation in urea Q was 44,100 calories mole⁻¹, ΔS was 61 calories degree⁻¹ mole⁻¹, and ΔF at 37° was 25,200 calories mole⁻¹. For the inactivation in 1 per cent NaCl, Q was 54,700 calories mole⁻¹, ΔS was 80 calories degree⁻¹ mole⁻¹, and ΔF at 37° was 29,800 calories mole⁻¹.

The inactivation of chorionic gonadotropin in 40 per cent aqueous urea solution to a product with 8 per cent of the original biologic activity had previously been studied. A repetition of these studies with a hormone with 3 times the biologic activity and prepared by a different chemical procedure gave essentially the same results, confirming the original deductions. The inactivation of the product with 8 per cent activity in 40 per cent aqueous urea solution in the temperature range 22.5–50.0° was also studied. The results indicated first order kinetics, but the low negative entropy of -17 calories degree $^{-1}$ mole $^{-1}$ and the heat of activation of 20,000 calories mole $^{-1}$ were within the range usually taken as representing normal reactions.

The inactivation of chorionic gonadotropin in 1 per cent NaCl solution was studied for the temperature range 50.0–65.0°. Inactivation rates were independent of concentration, indicating first order kinetics, but the rates were such that a first order reaction could only be assumed on the basis that the reaction product was a relatively stable substance with 24 per cent of the original biologic activity. The high heat of activation, 68,800 calories mole $^{-1}$, and high entropy of activation, 133 calories degree $^{-1}$ mole $^{-1}$, indicated that the process was a denaturation. Inactivation studies of chorionic gonadotropin at higher temperature (65–100°) indicated the possibility of a second reaction product with 10 per cent of the original biologic activity. The existence of a third product with 5 per cent of the original activity was definitely established, the velocity constant for the inactivation of this product at 99° being 3×10^{-2} min. $^{-1}$. The effects that this product produced upon ovarian and uterine tissues of immature rats were identical both qualitatively and quantitatively with that of the original hormone when the assay was based on the assumption that 5 per cent of the original activity survived in the heat-treated product.

The establishment of widely divergent inactivation rates constitutes the proof for the existence of biologically active denaturation derivatives of chorionic gonadotropin.

We wish to express our thanks to Dr. C. F. Church of The Squibb Institute for Medical Research for a sample of chorionic gonadotropin.

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ELECTROPHORETIC ANALYSES OF SERA AFTER TREATING DOGS WITH β -CHLOROETHYL VESICANTS*

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In a recent paper, Gilman and Philips (1) pointed out that the β -chloroethyl vesicants are extremely toxic compounds which have specific effects on the bone marrow, lymphoid tissues, and the intestinal mucosa. In addition, the systemic injury produced by these mustards causes a series of complex biochemical and physiological changes in the body. This investigation was undertaken to determine the effects of bis(β -chloroethyl) sulfide and three nitrogen mustards on the electrophoretic changes in the serum of dogs.

Methods

Dogs were fed a stock laboratory diet and were kept under observation for at least a month. The animals were in good condition at the beginning of the experimental period.

Redistilled sulfur mustard, bis(β -chloroethyl) sulfide, was administered by the following routes: (1) the whole animal or body (head excluded) exposed to known concentrations of vapor in specially constructed apparatus; (2) applied to shaved skin undiluted or dissolved in petroleum ether; (3) injected intravenously (saphenous) after dissolving in thiodiglycol. The hydrochlorides of ethylbis(β -chloroethyl)amine, methylbis(β -chloroethyl)amine, and tris(β -chloroethyl)amine were dissolved in saline immediately before intravenous injection (saphenous). Blood was drawn from the jugular, allowed to clot, and the serum withdrawn for analysis. Total nitrogen and non-protein nitrogen were determined by the micro-Kjeldahl procedure.

Serum was diluted with 1.5 parts of veronal buffer (μ , 0.1) at pH 8.6 and dialyzed against the buffer for 3 or 4 days. The electrophoretic experiments were done in a Tiselius apparatus at 2.0°, and the pattern recorded according to the scanning method of Longworth (2). The concentrations of the various protein fractions were estimated from the electrophoretic patterns obtained from the descending diagrams.

* This work was done under contract with the Medical Division of the Chemical Warfare Service.

Results

Fasting—Since the majority of dogs lose their appetite after treatment with the mustards, the serum proteins of a normal dog were studied electrophoretically before and after a 4 day period of fasting. The distribution of the serum components is not affected by fasting (Table I).

Vapor Exposure—Five dogs were exposed to high concentrations of sulfur mustard vapor. A typical example of the effect of this treatment

TABLE I
Electrophoretic Analysis of Serum of Fasting Dog

Days of fasting	Protein in 100 ml. serum					$\frac{\text{Albumin}}{\text{Globulin}}$
	Albumin	α -Globulin	β -Globulin	γ -Globulin	Total globulin	
	gm.	gm.	gm.	gm.	gm.	
0	3.24	1.35	0.83	0.61	2.79	1.15
4	3.17	1.23	0.82	0.70	2.75	1.14

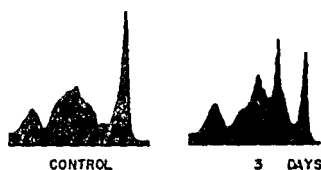


FIG. 1. Electrophoretic patterns for Dog 129 before and after the body was gassed with dichloroethyl sulfide.

TABLE II
Electrophoretic Analysis of Serum of Dog 129 Exposed to Sulfur Mustard Vapor

Days after exposure	Per cent of total area					$\frac{\text{Albumin}}{\text{Globulin}}$
	Albumin	α -Globulin	β -Globulin	γ -Globulin	Total globulin	
0	42.8	7.9	34.0	15.3	57.2	0.75
3	23.6	36.9	29.0	10.6	76.4	0.31

on the protein distribution of sera is shown in Table II and Fig. 1. This dog (No. 129) (head excluded) was exposed to a concentration of 500 γ per liter for 28 minutes ($Ct = 14,000$). Samples of blood were drawn before gassing and a few minutes before death on the 3rd day after treatment.

Because of insufficient sera, the nitrogen determinations could not be done. The relative changes in protein distribution were estimated from the percentage of the total area occupied by each fraction. The α -globulin fraction increased 350 per cent and albumin decreased 50 per cent.

Cutaneous Application—Mustard (50 mg. per kilo) was applied to the clipped skin of two dogs anesthetized with nembutal. Anesthesia was maintained for about 4 hours, and at the end of this time the agent remaining was removed with soap and water. The treated areas (about 10 sq. cm.) were edematous and blanched at this time. These animals were sacrificed on the 4th and 13th days after application of mustard owing to the poor condition of the animals.

The data for Dog 39, which was sacrificed on the 13th day, are shown in

TABLE III

Electrophoretic Analysis of Serum of Dog 39 after Cutaneous Application of Bis-(β -chloroethyl) Sulfide (50 Mg. per Kilo)

Days after treatment	Protein in 100 ml. serum				<u>Albumin</u> <u>Globulin</u>
	Albumin	α -Globulin	β -Globulin + γ -globulin	Total globulin	
	gm.	gm.	gm.	gm.	
Control	3.11	1.31	1.81	3.12	0.99
1	2.83	1.12	1.61	2.73	1.03
3	2.63	0.87	1.81	2.68	0.97
5	2.75	1.07	2.33	3.40	0.80
7	2.30	1.46	1.73	3.19	0.72
10	2.38	1.39	2.23	3.62	0.65
13	2.02	1.44	1.76	3.20	0.63

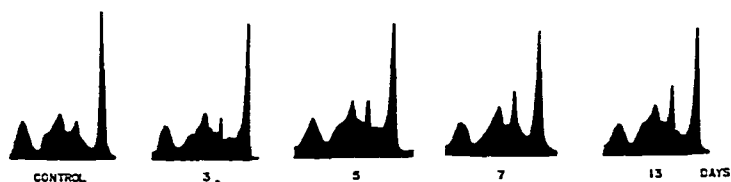


FIG. 2. Electrophoretic patterns for Dog 39 after application of 50 mg. per kilo of dichloroethyl sulfide on the skin.

Table III and Fig. 2. The albumin concentration decreased from a control level of 3.11 gm. per cent to a minimum of 2.02. The α , β , and γ fractions showed little change in concentration. However, examination of the electrophoretic pattern showed the presence of a pronounced spike in the α -globulin fraction. This unusual spike remained conspicuous for the entire period of observation. Despite the constancy of the globulin concentrations it is probable that a new protein fraction having the mobility of α -globulin has been added to the plasma.

Intravenous Injection of Sulfur and Nitrogen Mustards—Lethal doses of

bis(β -chloroethyl) sulfide were injected into two dogs. Electrophoretic data for the serum of Dog 11 are presented in Table IV and Fig. 3 to illustrate the effects of a non-lethal and a lethal dose of mustard in a single animal. After a sub-LD 50 dose (0.3 mg. per kilo), the protein distribution was not changed on the 1st day after this injection. This same dose was administered a week later and the animal survived 5 days. The albumin concentration was decreased but the globulins were not changed on

TABLE IV

Electrophoretic Analysis of Serum of Dog 11 after Two Injections of Bis(β -chloroethyl) Sulfide

Days after 1st injection (0.3 mg. per kilo)	Days after 2nd injection (0.3 mg. per kilo)	Protein in 100 ml. serum					Albumin Globulin
		Albumin	α -Globulin	β -Globulin	γ -Globulin	Total globulin	
		gm.	gm.	gm.	gm.	gm.	
Control		2.8	0.98	1.44	0.53	2.95	0.95
1		2.7	0.75	1.67	0.67	3.09	0.88
7				1.04	0.75		
	1	2.2	0.79	1.30	0.67	2.76	0.81
	3	1.9	1.67	1.57	0.58	3.82	0.49
	4	1.9	2.47	1.00	0.62	4.09	0.47

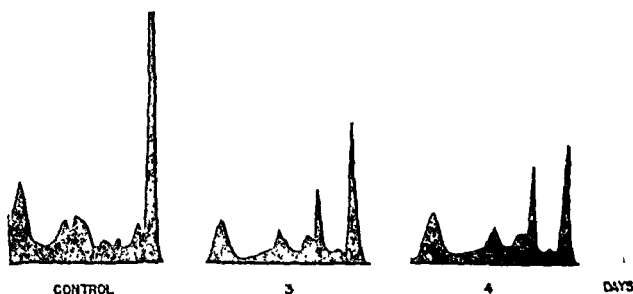


FIG. 3. Electrophoretic patterns for Dog 11 after the intravenous injection of dichloroethyl sulfide.

the 1st day after the second injection. On the 3rd and 5th days, the albumin decreased further and the α -globulin concentration rose markedly. The β -globulin concentration decreased slightly and the γ fraction showed little change. The albumin to globulin ratios decreased from the control value of 0.95 to 0.47.

Five dogs were injected with varying doses of ethylbis(β -chloroethyl)-amine hydrochloride. The electrophoretic patterns of animals receiving LD 100 doses were identical. After injection of sub-LD 50 doses, no sig-

nificant changes in protein were seen. After the injection of 2.0 mg. per kilo in Dog 44, severe vomiting and diarrhea occurred on the 3rd day, and the animal died on the 4th day.

The electrophoretic patterns of all animals in this group were unsatisfactory and difficult to analyze. These particular studies were carried out during particularly hot weather, which may be associated with the type of pattern obtained.

A typical spike appeared in the α -globulin region. The total globulin concentration increased from 1.64 to 3.96 gm. per cent. The albumin

TABLE V
Electrophoretic Analysis of Serum of Dog 44 Injected Intravenously with 2.0 Mg. per Kilo of Ethylbis(β -chloroethyl)amine Hydrochloride

Days after treatment	Protein in 100 ml. serum				<u>Albumin</u> <u>Globulin</u>
	Albumin	α -Globulin + α -globulin	β -Globulin + γ -globulin	Total globulin	
	gm.	gm.	gm.	gm.	
Control	3.46			1.64	2.10
3	2.28	1.19	1.94	3.13	0.72
4	2.08			3.96	0.52

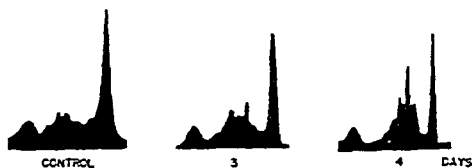


FIG. 4. Electrophoretic patterns for Dog 44 after the intravenous injection of ethylbis(β -chloroethyl)amine.

concentration decreased from 3.46 to 2.08 gm. per cent. These changes caused a reversal of the albumin to globulin ratio (Table V and Fig. 4).

LD 100 doses of methylbis(β -chloroethyl)amine hydrochloride were injected into three dogs. The data for Dog 46, which survived for 4 days, are shown in Table VI and Fig. 5. This animal exhibited characteristic diarrhea and vomiting which follows injection of the nitrogen mustards. The typical decrease in albumin, increase in α -globulin, and decrease in the albumin to globulin ratio are seen.

Tris(β -chloroethyl)amine hydrochloride was injected in varying amounts into five dogs. The data for Dog 15, which survived 3 days, are presented in Table VII and Fig. 6. The albumin concentration of these sera showed no change in concentration. This constancy was not observed in any

TABLE VI

Electrophoretic Analysis of Serum of Dog 46 Injected Intravenously with 1.25 Mg. per Kilo of Methylbis(β -chloroethyl)amine Hydrochloride

Days after treatment	Protein in 100 ml. serum				$\frac{\text{Albumin}}{\text{Globulin}}$
	Albumin	α_1 -Globulin + α_2 -globulin	β -Globulin + γ -globulin	Total globulin	
	gm.	gm.	gm.	gm.	
Control	3.50	0.84	1.68	2.52	1.38
1	3.36	1.19	1.41	2.60	1.23
3	3.16	1.59	1.32	2.91	1.03
4	2.63	2.39	1.67	4.06	0.64

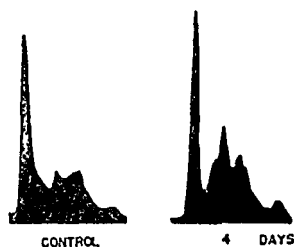


FIG. 5. Electrophoretic patterns for Dog. 46 after the intravenous injection of methylbis(β -chloroethyl)amine.

TABLE VII

Electrophoretic Analysis of Serum of Dog 15 Injected Intravenously with 1.5 Mg. per Kilo of Tris(β -chloroethyl)amine Hydrochloride

Days after treatment	Protein in 100 ml. serum				$\frac{\text{Albumin}}{\text{Globulin}}$
	Albumin	α -Globulin	β -Globulin + γ -globulin	Total globulin	
	gm.	gm.	gm.	gm.	
Control	3.13	0.64	2.50	3.14	1.00
1	3.30	0.79	2.62	3.41	0.97
2	3.30	1.15	1.87	3.02	1.09
3	3.27	1.30	1.73	3.03	1.08

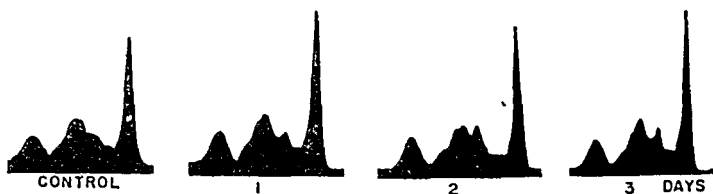


FIG. 6. Electrophoretic patterns for Dog 15 after the intravenous injection of tris(β -chloroethyl)amine hydrochloride (1.5 mg. per kilo).

other treated animal. The α -globulin was increased. The data for β - and γ -globulins were combined owing to poor separation in the pattern.

Comment

Although the changes in the albumin and α -globulin concentrations are consistently noted after treatment with noxious substances, no evidence is available to indicate the mechanisms involved. In the case of bis(β -chloroethyl) sulfide, cutaneous treatment with the vapor and liquid or intravenous injection produced the same type of electrophoretic changes. The four mustards used are structurally related by having the chloroethyl radical in each molecule. A common denominator for these mustards is their cytotoxic properties regardless of the mode of administration.

These studies indicate that there is a latent period of 2 or 3 days before the electrophoretic pattern shows the definite and marked changes described for α -globulin. Unpublished data indicate that the increase in the globulin area is due to the presence of several new protein fractions; one is extremely rich in lipides and another is poor in lipides. The greatest increase in the α -globulin concentration of this series was noted in a dog exposed to a lethal concentration of vapor. Body exposure is followed by extensive damage to the skin of the entire animal. Intravenous injection of LD 100 doses of the sulfur and nitrogen mustards produces changes which are qualitatively and quantitatively similar. Dogs surviving one or more non-lethal doses show no significant changes in protein distribution.

Inspection of the electrophoretic patterns shows that the β -globulin area is characterized by the appearance of a spike. Although this change is not conspicuous, it appears to be present in varying degrees in the sera of the intoxicated animals. The changes in the percentage concentrations of the β -globulins are not striking. On the basis of unpublished fractionation experiments, it is believed that the spike seen in the β -globulin area represents the addition of new proteins to the plasma. In some of the sera, similar spikes are observed for the α -globulin fractions without a change in the percentage concentration.

The mustards used in these experiments cause a very marked lymphopenia in rats, rabbits, and dogs after the first 24 hours. Marked destruction and dissolution of the lymphoid tissues occur in these species. White and Dougherty (3) found that the total serum proteins increase slightly shortly after the injection of pituitary adrenotropic hormone in rats. This change is related to the destruction of lymphocytes following the injection. In a later paper, these workers (4) showed that both the β - and γ -globulins increase in normal rabbits during the 1st day following injections of either adrenotropic hormone or adrenal cortical steroids. These and other findings have been interpreted to mean that lymphoid tissue is a source of

globulins. The electrophoretic analyses of the sera of dogs treated with mustards do not appear to confirm the ideas expressed by the Yale investigators, since the areas of the β - and γ -globulins are not increased. However, there is some indication that the response to injury, as evidenced by the electrophoretic pattern of serum, is different in the dog and rabbit.

SUMMARY

1. Serum proteins of dogs were analyzed after exposing the skin to vapor and liquid bis(β -chloroethyl) sulfide and after the intravenous injection of bis(β -chloroethyl) sulfide and the hydrochlorides of ethylbis(β -chloroethyl)-amine, methylbis(β -chloroethyl)amine, and tris(β -chloroethyl)amine.

2. Exposure to or injection of LD 100 doses of these agents causes a decrease in the albumin and an increase in the α -globulin concentration of sera.

3. The greatest changes in the albumin and α -globulin were seen after exposure of the body to bis(β -chloroethyl) sulfide vapor. Injection of LD 100 doses of the sulfur and nitrogen mustards causes approximately the same quantitative changes in the electrophoretic patterns.

4. Characteristic spikes in the α and β areas characterize the electrophoretic patterns of the serum of the treated dog.

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ELECTROPHORETIC ANALYSES OF SERA OF INJURED DOGS*

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Severe injury to dogs by sulfur and nitrogen mustards causes a decrease in albumin and an increase in the α -globulin concentrations of the serum as measured by the electrophoretic procedure (1). These changes in the distribution of proteins appear to be a manifestation of a complex series of metabolic disturbances which occur after extensive cellular death. Little is known concerning the nature of the products of tissue destruction responsible for these disturbances.

This investigation was undertaken to observe the effects of a wide variety of injury on the protein distribution in the serum.

Methods and Results

Healthy male and female dogs were used as experimental animals. The procedures for electrophoresis have been described (1). Injury was produced by heat, cold, turpentine, and bone fracture under anesthesia.

Hot Water—Anesthetized dogs (nembutal), whose hair was clipped, were burned by dipping in water at 73° for 6 seconds. The head, shoulders, and fore legs were not exposed to the hot water. The genitalia were protected with wet gauze. Three dogs were treated in this manner.

The changes in the serum of Dog 26 are representative for the effect of scalding and the data are shown in Fig. 1 and Table I. At the end of the first 24 hours, the hind legs were edematous and several necrotic patches were noted on the inner surface of the legs. At this time, the albumin concentration decreased from the control value of 3.31 to 1.31 gm. per cent; the α -globulin fraction increased from 0.29 to 1.03 gm. per cent. On the 2nd and 3rd days, the hind legs were very edematous and the skin was covered with numerous small blisters and necrotic patches. The α -globulin concentrations continued to increase on the 2nd and 3rd days and were 1.36 and 1.63 gm. per cent, respectively; the albumin concentration rose to 1.89 gm. per cent on the 3rd day. The α - and β -globulins showed characteristic spikes on the 3rd day.

The albumin concentration reached a level of about 2.0 gm. per cent on the 4th day, which was maintained. The α -globulin fraction continued to increase to a maximum of 2.0 gm. per cent on the 7th day. The β and γ

* This work was done under contract with the Medical Division of the Chemical Warfare Service.

fractions could not be analyzed separately owing to poor electrophoretic separation. The dog was sacrificed on the 7th day owing to the large areas of skin necrosis.

Dry CO₂—A large number of experiments were conducted to determine the procedures for effectively damaging skin with dry ice. It was found that the application of dry ice to the inner aspects of the back and front legs for 3 minutes destroyed the areas so treated. Dog 27 was severely burned by the above procedure. 1 day later, the skin was blistered and showed

TABLE I

Electrophoretic Analysis of Serum of Dog 26 after Burning with Water at 73° for 6 Seconds

Days after burning	Protein in 100 ml. serum					Albumin Globulin
	Albumin	α -Globulin	β -Globulin	γ -Globulin	Total globulin	
	gm.	gm.	gm.	gm.	gm.	
Control	3.31	0.29		2.00	2.29	1.43
1	1.31	1.03		1.17	2.20	0.59
2	1.42	1.36		1.40	2.76	0.51
3	1.89	1.63		1.68	3.31	0.57
4	2.01	1.97		1.58	3.55	0.57
5	2.01	1.90	1.42	0.28	3.60	0.56
7	1.91	2.00		1.62	3.62	0.53

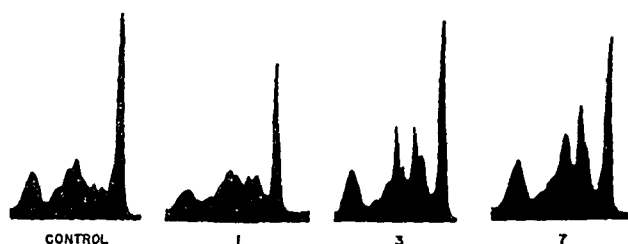


FIG. 1. Electrophoretic patterns for Dog 26 after being burned with hot water under anesthesia at 73° for 6 seconds.

some signs of weeping. On the 2nd day, there was considerable weeping and some necrosis. Owing to severe sloughing and signs of infection, the animal was sacrificed on the 5th day.

The electrophoretic patterns and analyses are shown in Fig. 2 and Table II. At the end of the 1st day, the albumin concentration was almost halved and the α -globulin concentration was doubled. The concentration of β -globulin, which was characterized by a spike, could not be determined. During the remainder of the experiment, the albumin concentration re-

mained low, the α -globulin fraction increased to 220 per cent above the control, and the β -globulin spike persisted. The albumin to globulin ratio was increased considerably.

Turpentine—This material was injected subcutaneously in 0.5 ml. quantities into six different sites on the backs of two dogs. Edema, inflammation, and some necrosis were seen on the 3rd day. Sloughing began on the 4th day. These animals were sacrificed on the 7th (Dog 32) and the 11th (Dog 31) days owing to infection of the necrotic areas.

The patterns for Dog 32 (Fig. 3) showed the characteristic spikes for the α - and β -globulins. The decrease in albumin and the increase in α -globulin

TABLE II

Electrophoretic Analysis of Serum of Dog 27 after Burning with Dry Ice (CO₂)

Days after burning	Protein in 100 ml. serum				$\frac{\text{Albumin}}{\text{Globulin}}$
	Albumin	α -Globulin	β -Globulin + γ -globulin	Total globulin	
	gm.	gm.	gm.	gm.	
Control	3.33	0.65	1.12	1.77	1.86
1	1.86	1.25	0.99	2.24	0.83
3	1.62	2.10	0.95	3.05	0.53
5	1.90	2.00	0.85	2.85	0.67

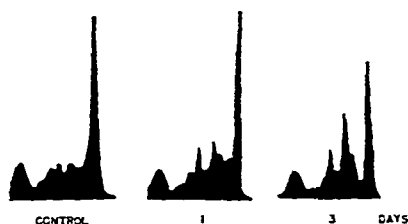


FIG. 2. Electrophoretic patterns for Dog 27 after being burned with dry ice for 3 minutes.

concentrations (Table III) were pronounced during the experimental period. The greatest changes were seen on the 3rd day after injection.

Bone Fracture—Under nembutal anesthesia the tibia of the left leg of Dog 56 (Table IV) was fractured under sterile conditions, through a small skin incision by means of an osteotome. A cast was applied immediately.

The albumin concentration did not change during the 17 day period of observation. Both the α_1 - and α_2 -globulin fractions increased and the greatest change was noted on the 3rd day. The combined β and γ fractions remained fairly constant. It should be emphasized that the trauma was minimal.

Skin Trauma—A dog was anesthetized and approximately 120 sq. cm. of abdominal skin were subjected to squeezing in a vise and by clamps. The crushed skin was paper-thin and some vesication was present immedi-

TABLE III

Electrophoretic Analysis of Serum of Dog 32 Injected Subcutaneously with 3.0 Ml. of Turpentine

Days after treatment	Protein in 100 ml. serum					Albumin Globulin
	Albumin	α -Globulin	β -Globulin	γ -Globulin	Total globulin	
	gm.	gm.	gm.	gm.	gm.	
Control	2.85	1.30		1.75	3.05	0.93
3	1.61	2.13		1.75	3.88	0.41
5	1.74	1.78	1.40	0.49	3.67	0.47
7	1.84	1.54	1.45	0.67	3.66	0.50

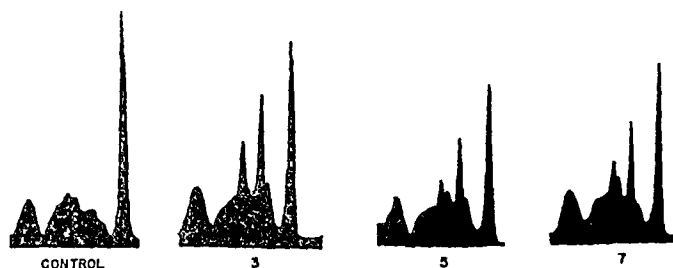


FIG. 3. Electrophoretic patterns for Dog 32 after the subcutaneous injection of 3.0 ml. of turpentine.

TABLE IV

Electrophoretic Analysis of Serum of Dog 56 after Bone Fracture

Days after treatment	Protein in 100 ml. serum					Albumin Globulin
	Albumin	α_1 -Globulin	α_2 -Globulin	β -Globulin + γ -globulin	Total globulin	
	gm.	gm.	gm.	gm.	gm.	
Control	2.67	0.28	0.46	2.97	3.71	0.71
1	2.30	0.59	0.68	2.73	4.00	0.57
3	2.39	0.48	1.04	2.60	4.12	0.57
8	2.65	0.38	0.70	2.68	3.76	0.70
10	2.51		1.48	2.31	3.79	0.65
17	2.56		0.94	2.59	3.54	0.72

ately after the injury. The skin healed rapidly and the only sign of damage on the 4th day was a slight erythema. The distribution of the serum proteins was not affected during the 4 day period of observation.

DISCUSSION

The present experiments demonstrate that injury causes changes in the distribution of the plasma proteins. Various types of injury, whether due to chemical or physical factors, appear to cause a decrease in the albumin, an increase in the α -globulin fraction, and a change in the appearance of the β -globulin pattern. Similar changes in the electrophoretic patterns are known to occur in a large variety of conditions involving inflammation or tissue destruction in patients (2-6).

The present study stresses the probability that the destruction and subsequent disintegration of tissue are directly responsible for the formation of α -globulin. This appears to be borne out by the latent period of several days before the α -globulin appears in appreciable amounts and by the appearance of spikes in the electrophoretic pattern in the serum of an injured animal. Work in progress indicates that at least one or possibly more new proteins, which have the mobility of α -globulin, appear in the blood. It would be interesting to determine, if possible, whether or not injury to various tissues would cause the formation of a variety of new plasma proteins.

A decrease in albumin concentration is the earliest change seen in the serum after injury. This is probably due to a loss of albumin through damaged capillaries. Marked decreases are seen after thermal injury, exposure to mustard vapor, and turpentine injections in which large areas of skin are destroyed.

The increased α -globulin concentration in the plasma of injured animals may be associated with the formation of physiologically active materials. Menkin (7) discusses the presence of a number of active materials; *i.e.*, leucotaxine, leucocytosis-promoting factor, necrosin, and pyrexin in globulin fractions of inflammatory exudates. Smith and Smith (8) describe the presence of a fibrinolytic enzyme and a toxic material which are associated with globulin fractions of menstrual fluid and the blood of menstruating women. The sera of patients with late pregnancy toxemia, eclampsia, and gynecological complaints are also fibrinolytic. These workers suggest that a proteolytic enzyme is produced by injured tissues.

SUMMARY

1. The serum proteins of dogs were analyzed electrophoretically before and after injury by (1) hot water, (2) cold (dry CO_2), (3) subcutaneous injection of turpentine, and (4) bone fracture.

2. The albumin concentration decreased and the α -globulin fraction increased. Sharp spikes appearing in the α and β areas are interpreted as an indication for the appearance of abnormal proteins in the serum.

3. It is concluded that severe tissue destruction in the dog causes marked changes in the distribution and type of proteins in the blood plasma.

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URINARY PHENOLS

II. EFFECT OF DIET ON THE PHENOLIC BODIES OF THE URINE*

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During recent years renewed interest has been evinced in the phenolic bodies in the blood and urine (1-6). Thus abnormalities in the metabolism of phenolic compounds have been reported in connection with pernicious anemia (7), phenylpyruvic oligophrenia (8-9), tyrosinosis (10), premature infants (11), ascorbic acid utilization (12), hepatic function (13), etc. Recently an improved method for urinary phenols was developed in this laboratory (14). By means of ether extraction at selective pH, more accurate and specific values for the urinary phenolic bodies can be obtained than with the older methods of Folin and Denis (15) and Theis and Benedict (16). The present communication is concerned with a study of the influence of diet upon the phenolic and nitrogenous constituents of the urine as determined by the more specific methods.

EXPERIMENTAL

Methods and Materials—The various phenolic bodies were estimated by ether extraction and diazotization methods previously described (2). Free and total "phenols" were also determined by the less specific method of Folin and Denis (15). Total nitrogen was determined colorimetrically after oxidation of 1 cc. of 1:100 diluted urine and nesslerization. Values for amino acid nitrogen were obtained by the method of Sahyun (17) after slight modifications.¹

Influence of Diet—In the first experiment three young women and one man (age 36) volunteered to go on an average diet for 3 days. They were then placed on a low protein-high carbohydrate diet for 3 more days and finally on a high protein-low carbohydrate regimen for 4 days.

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¹ 5 cc. of urine were diluted to 50 cc. with distilled water, treated successively three times with 2.5 gm. portions of permutit, and then centrifuged, as the ammonia-containing permutit reacts with the naphthoquinone reagent. Excess alkali causes considerable irregularity in color production; hence final addition of NaOH was made with 0.01 N NaOH to a light pink to phenolphthalein.

24 hour urine specimens were collected and preserved in the cold shortly after voiding. The analytical data are given in Table I. The degree of effectiveness of the diets is revealed in the reduction in total nitrogen from an average high of 11.2 gm. to a low of 4.7 gm. (58 per cent) per 24 hour specimen. A rise to 15.4 gm. (69.5 per cent) then followed the high protein intake. The amino acid nitrogen followed a similar although somewhat less marked pattern.

Although each of the four individuals showed different levels of excretion for the various urinary phenolic bodies, all values are within the normal range established in previous work (14). Since individual

TABLE I

Effect of Diet on Phenolic and Nitrogenous Constituents of Urine

All values by the ether extraction methods are reported as mg. of phenol-p-cresol per 24 hour specimen, and amino acid nitrogen as mg. and total nitrogen as gm. per 24 hour specimen; by the Folin-Dennis method, as mg. of phenol per 24 hour specimen.

Specimen No.	Diet	Urine	Urinary phenols		Aromatic hydroxy acids		Total phenolic bodies	Phenols by Folin-Denis method		Amino acid N	Total N
			Free	Conjugated	Free	Conjugated		Free	Conjugated		
1	Average	1000	1.3	39	71	47	158	332	102	142	9.1
2	"	1070	1.4	34	75	28	138	276	97	117	11.1
3	"	973	1.3	40	80	33	154	288	98	146	9.6
4	Low protein	917	2.2	27	66	44	139	289	91	138	11.2
5	" "	870	1.2	25	70	38	134	251	81	111	6.3
6	" "	900	2.0	34	76	41	153	280	75	100	4.7
7	High "	977	1.7	32	83	31	148	299	72	120	8.5
8	" "	1010	1.7	45	63	33	143	293	87	145	12.5
9	" "	950	1.7	37	84	45	168	335	101	159	15.4
10	" "	1000	1.3	44	74	49	168	311	105	151	14.7

responses to the various diets were essentially alike, only the average values for each constituent for the four experiments are reported. The data in Table I indicate, however, that the output of urinary phenolic bodies was not markedly influenced by the dietary changes. While conjugated and total phenols seemed to be depressed slightly by the low protein diet, the concentrations of free and conjugated aromatic hydroxy acids were not influenced by either the low or the high protein diets. Values for the total phenolic bodies were not appreciably altered except for a slight elevation following several days of high protein ingestion. "Phenols" by the Folin-Denis method were found to decrease slightly

and then to rise again when protein was ingested in larger amounts. These changes are due in part to variations in the concentration of amino acids, since this method is sensitive to tyrosine, histidine, etc.

In the next experiment a young man, following an average diet, had adequate caloric intake supplied exclusively by glucose for 3 days. This regimen was followed by a high carbohydrate-low protein intake for 3 days and finally by a high protein diet for 4 more days. The analytical data in Table II indicate that urinary total nitrogen and amino acid nitrogen decreased and then rose to the high levels as expected. While the output of

TABLE II

Effect of Diet on Phenolic and Nitrogenous Constituents of Urine

All values by the ether extraction methods are reported as mg. of phenol-*p*-cresol per 24 hour specimen, and amino acid nitrogen as mg. and total nitrogen as gm. per 24 hour specimen; by the Folin-Denis method, as mg. of phenol per 24 hour specimen.

Specimen No.	Diet	Urine	Urinary phenols		Aromatic hydroxy acids		Total phenolic bodies	Phenols by Folin-Denis method		Amino acid N	Total N
			Free	Conjugated	Free	Conjugated		Free	Conjugated		
		cc.									
1	Average	1420	1.0	49	92	48	190	435	145	237	17.2
2	"	1300	0.9	54	79	51	185	433	132	217	12.6
3	Glucose	1890	0.8	55	85	38	179	270	101	187	8.9
4	"	1260	0.5	70	26	24	121	197	78	136	8.2
5	"	1020	0.7	74	53	27	155	204	115	166	8.5
6	Low protein	1000	0.7	67	67	48	183	269	116	159	6.8
7	" "	1300	0.9	51	120	59	231	342	122	197	8.1
8	" "	1600	0.8	61	117	38	217	324	116	217	10.0
9	High "	1630	0.7	55	115	78	246	366	147	222	8.1
10	" "	1250	0.9	81	203	65	355	450	137	204	17.5
11	" "	1120	1.4	100	158	94	352	473	149	207	19.4
12	" "	1010	1.2	84	134	60	279	439	192	232	17.3

free and conjugated phenols was not significantly altered by the variations in the diet, a marked decrease in free aromatic hydroxy acids, from 92 to 26 mg. of phenol-*p*-cresol (72 per cent), in conjugated aromatic hydroxy acids, from 51 to 24 mg. (54 per cent), and in total acids, from 140 to 50 mg. (65 per cent), occurred upon the exclusive glucose intake. These values, however, returned to normal levels even with a low protein diet. However, in this experiment, the output of both phenol and aromatic hydroxy acids was considerably increased above normal by the high protein diets. It seems obvious that drastic dietary regimens must be employed

in order to produce changes in phenolic output beyond the ordinary normal range. Values by the Folin-Denis method decreased in response to the decline in aromatic hydroxy acids and amino acids resulting from the low protein intake.

SUMMARY

1. The influence of protein intake upon the output of phenolic and nitrogenous constituents of the urine, as measured by more specific methods, has been studied.

2. Significant changes in the concentration of urinary phenolic bodies beyond the normal range of values was not observed unless protein was rigidly eliminated from the diet.

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URINARY PHENOLS

III. EFFECT OF DIET AND PHTHALYLSULFATHIAZOLE ON THE URINARY PHENOLS OF THE DOG*

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Urinary phenols are believed to be products of exogenic origin, formed by the putrefactive action of intestinal bacteria on the aromatic amino acids. The influence of relatively insoluble sulfonamides on the bacterial flora of the intestinal tract has stimulated considerable interest (1-3) and the biochemical response to these antibiotics has been duly reported (4-8). In the present work, the influence of diet and also phthalylsulfathiazole (sulfathalidine) on the urinary phenolic bodies of the dog was investigated.

EXPERIMENTAL

Influence of Diet—A young, male dog weighing about 10 kilos, was placed in a metabolism cage and given an adequate amount of a commercial dog ration (Kasco brand) twice daily. Water was available at all times. The feces were removed, whenever possible, shortly after defecation, in order to minimize contamination. The urine was collected several times daily and preserved in the refrigerator. Each 48 hour specimen was mixed and centrifuged to remove extraneous material. This routine was continued for 10 days until the concentration of the various urinary constituents had attained reasonably constant values. Then a protein-free solution containing adequate amounts of sucrose, electrolytes, and vitamins (9) was given for 6 days. The dog ate these sugar solutions with relish. The urine was analyzed for the various phenolic fractions, amino acid nitrogen, total nitrogen, etc., by methods outlined in Paper II.

The data, given in Table I, show that the output of phenolic bodies, amino acids, and total nitrogen decreased greatly immediately upon ingestion of the protein-free sugar solutions. Thus the free phenols decreased from 2.0 to 0.37 mg. of phenol-*p*-cresol per 48 hour specimen (81 per cent), conjugated phenols from 11.8 to 6.0 mg. (49 per cent), free ether-soluble aromatic hydroxy acids from 93 to 25 mg. (73 per cent), and conjugated aromatic hydroxy acids from 112 to 33 mg. (71 per cent), respectively. Phenols by the Folin-Denis (10) method, amino acid nitrogen, and total

* Aided by a grant from the Bressler Alumni Research Fund.

† John F. B. Weaver Fellow in Biological Chemistry.

TABLE I

Effect of Diet on Phenolic and Nitrogenous Constituents of Urine of Dog

All values by the ether extraction methods are reported as mg. of phenol-*p*-cresol per 48 hour specimen, and amino acid nitrogen as mg. and total nitrogen as gm. per 48 hour specimen; by the Folin-Denis method, as mg. of phenol per 48 hour specimen.

Specimen No.	Diet	Urine	Urinary phenols		Aromatic hydroxy acids		Total phenolic bodies	Phenols by Folin-Denis method		Amino acid N	Total N
			Free	Conjugated	Free	Conjugated		Free	Conjugated		
		cc.									
1	Kasco ration	500	2.6	10.5	65	76	154	352	124	215	11.4
2	" "	575	2.5	8.3	63	83	157	384	148	266	13.0
3	" "	465	2.1	8.7	72	94	177	455	162	296	12.4
4	" "	500	1.9	10.1	93	103	208	470	147	285	13.0
5	" "	555	2.0	11.8	90	112	216	535	145	340	15.8
6	Sucrose solution	450	1.1	7.0	43	54	105	250	59	132	4.0
7	" "	850	0.7	7.0	30	39	77	276	57	145	3.3
8	" "	1350	0.4	6.0	25	33	61	260	47	118	2.0

TABLE II

Effect of Phthalylsulfathiazole on Phenolic and Nitrogenous Constituents of Urine of Dog

All values expressed per 48 hour specimen; values by the ether extraction methods as mg. of phenol-*p*-cresol and by the Folin-Denis method as mg. of phenol.

Specimen No.	Urine	Total phenols	Aromatic hydroxy acids		Total phenolic bodies	Phenols by Folin-Denis method		Amino acid N	Total N	Sulfathiazole in urine		Coliform organisms per gm. wet stool
			Free	Conjugated		Free	Conjugated			Free	Total	
	cc.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	gm.	mg.	mg.	
1	700	12	134	92	238	458	159	244	12.0			2×10^{10}
2	800	12	125	111	248	508	142	265	12.2			2×10^{10}
Gave drug												
3	900	11	113	87	211	426	136	250	12.2	118	266	1×10^5
4	1300	10	90	93	193	376	112	213	13.1	315	741	None
5	950	8	114	100	222	500	135	250	12.0	320	750	"
6	475	7	119	87	213	376	116	213	11.6	315	740	"
7	780	7	97	90	194	373	125	249	12.8	315	697	"
8	600	6	98	84	188	344	150	261	12.5	338	800	"
Discontinued drug												
9	500	6	102	91	199	382	158	249	13.1	250	425	
10	600	7	84	84	175	312	143	249	12.0	2	3	
11	500	10	84	99	193	281	127	230	14.3			
12	707	10	120	94	224	435	143	242	13.0			
13	630	13	121	118	252	526	141	227	12.8			

nitrogen decreased markedly as expected. The data also indicate that, following ingestion of the sucrose diet for several days, the output of phenolic and nitrogenous bodies tends to level off and remain relatively constant. Whether this is the actual minimum level of formation and excretion, however, can only be proved by more extensive and prolonged experiments. While there is some indication that a portion of these phenolic bodies may have an endogenous origin, the evidence is not conclusive. It is interesting to note that the urine of the dog on the Kasco diet, calculated on a 24 hour basis, contained fewer phenols than have been previously reported for human urine (11). However, both urines contained approximately the same amounts of aromatic hydroxy acids.

Influence of Phthalylsulfathiazole on Urinary Phenolic Bodies—The administration of poorly adsorbed sulfonamides to alter the bacterial flora of the intestinal tract has become a well established clinical procedure. Poth and Ross (3) found that the *Clostridia* and coliform bacteria are reduced to very low levels in the feces of the dog and that all anaerobic vegetative organisms disappear within 48 hours after administration of phthalylsulfathiazole. However, the drug was found to be ineffective against *α-Streptococcus fecalis*, *Bacillus proteus*, and the typhoid and paratyphoid organisms. Hence the feces never become completely sterile. This drug does not produce liquid stools and is also more effective than succinylsulfathiazole in altering the coliform flora.

The dog was given 10 gm. of the phthalylsulfathiazole mixed with an adequate amount of the ration (Kasco) every 24 hours. The feedings were divided into six equal 4 hour periods and continued for 12 days. The 48 hour urine specimens were preserved in the cold and subjected to the usual analyses. The presence of sulfonamides introduced certain analytical complications¹ since they react with the diazotized *p*-nitroaniline reagents used in the phenol determinations. Immediately after defecation the stool

¹ While the presence of the sulfonamides in the urine does not interfere with the Folin-Denis method for phenols and only slightly with that for amino acid nitrogen, it does complicate the procedures involving ether extraction, since some of the drug is slowly extracted by the ether and comes out of solution in the receiver. However, it goes back into solution again upon the addition of alcohol. Therefore an aliquot of the ether-alcohol extract was evaporated to dryness, taken up in water containing 8 per cent *p*-toluenesulfonic acid, and analyzed for free and total sulfathiazole by the Bratton-Marshall method (12). Ether extracts of sulfonamide-free urines yield no appreciable color with these reagents. By colorimetric determinations at various concentrations it was found that 1 part of the phenol-*p*-cresol mixture yields as much color with the diazotized *p*-nitroaniline as 8 parts of sulfathiazole or acetylsulfathiazole. While the color given by the sulfonamides in the ether-alcohol extract of the urines was not excessive, the amount was deemed necessary for correction. This was effected by adding an amount of sulfathiazole to the phenol-*p*-cresol standard equivalent to that found by analysis in the ether extracts.

specimens were studied bacteriologically by the general procedure outlined by Poth and Knotts (2) and in a private communication from the Sharp and Dohme laboratories.²

The chemical and bacteriological data are given in Table II. The number of coliform bacteria rapidly decreased to negligible amounts shortly after administration of phthalylsulfathiazole. However, the concentration of the various phenolic bodies in the urine was not greatly influenced by this change in fecal flora. While a moderate decrease in urinary phenols was observed, the formation and output of aromatic hydroxy acids and total phenolic bodies were not markedly influenced by the ingestion of the sulfonamide. Obviously, the administration of the protein-free sucrose solution was far more effective in reducing the concentration of the various phenolic bodies in the urine. The amino acid and total nitrogen content of the urine remained relatively unchanged during the experiment. Since the feces are not completely sterilized by phthalylsulfathiazole, it seems apparent that most of the phenol-producing organisms are not inhibited by this sulfonamide.

We are indebted to Sharp and Dohme, Inc., Philadelphia, for a generous gift of phthalylsulfathiazole and to Dr. F. Hachtel of the Department of Bacteriology of this school for helpful cooperation in the bacteriological studies.

SUMMARY

1. The ingestion of a protein-free diet of sucrose, salts, and vitamins resulted in a marked decrease in the urinary phenolic bodies of the dog.
2. The addition of phthalylsulfathiazole to a commercial dog ration did not reduce greatly the formation and excretion of phenolic bodies in the urine.

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²A small quantity of the fresh fecal material was picked up with a sterile glass rod and deposited in the bottom of a sterile 15 cc. glass graduated centrifuge tube. The fecal material (0.3 to 0.6 cc. in volume) was rubbed up with 2 cc. of sterile water and centrifuged at about 2500 r.p.m. for 20 minutes. Sterile water was added to a total volume of 10 times the volume of fecal solids and thoroughly emulsified. This was the 1×10^{-1} dilution. Subsequent 10-fold dilutions were prepared to 1×10^{-7} . 1 cc. portions were plated on poured desoxycholate agar plates. 5 mg. per cent of *p*-aminobenzoic acid were added to all culture media. Counts of all red-colored colonies after 24 hours incubation gave the coliform content of the stool specimens.

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USE OF SULFASUXIDINE, STREPTOTHRICIN, AND STREPTOMYCIN IN NUTRITIONAL STUDIES WITH THE CHICK*

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Sulfonamides are known to alter the intestinal flora of animals, with a consequent change in the synthesis of certain vitamins by the intestinal bacteria (1-5). This altered synthesis may in turn lead to an increased dietary vitamin requirement for some animals or invoke new requirements in others. For example, the inclusion of 1 per cent of sulfasuxidine in the diet of chicks increases their folic acid requirement about 3-fold (6).

The question then naturally arises whether other bactericidal or bacteriostatic agents could produce still different changes in the intestinal flora and lead to increased requirements for other vitamins or to new types of deficiencies. Further, a reverse phenomenon could possibly be encountered; namely, the inhibition of certain bacterial groups which might decrease the growth of the animal either through the consumption and consequent immobilization of vitamins or through the production of toxic compounds. Finally, a drug or combination of drugs that would completely inactivate all bacteria in the intestinal tract would be highly desirable, since investigators would be provided with an essentially sterile animal and vitamin requirements could be studied uncomplicated by "intestinal vitamins" or toxic substances. This investigation was carried out with the latter view in mind.

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We are indebted to Merck and Company, Inc., Rahway, New Jersey, for the crystalline vitamins and streptothricin; to Wilson and Company, Inc., Chicago, for gelatin; to The Wilson Laboratories, Chicago, for liver fraction L and whole liver substance; to Allied Mills, Inc., Peoria, Illinois, for soy bean oil; to Dr. B. L. Hutchings of the Lederle Laboratories, Inc., Pearl River, New York, for synthetic folic acid; to Sharp and Dohme, Inc., Philadelphia, Pennsylvania, for sulfasuxidine; to the Abbott Laboratories, North Chicago, Illinois, for haliver oil; to E. I. du Pont de Nemours and Company, Inc., New Brunswick, New Jersey, for crystalline vitamin D₂; and to Dr. J. M. McGuire of Eli Lilly and Company, Indianapolis, Indiana, for streptomycin.

EXPERIMENTAL

Day-old white Leghorn cockerels obtained from a commercial hatchery were maintained in electrically heated cages with raised screen bottoms and fed a basal ration consisting of dextrin 61 gm., alcohol-extracted casein 18 gm., gelatin 10 gm., Salts 5 (7) 6 gm., soy bean oil 5 gm., *l*(-)-cystine 300 mg., *i*-inositol 100 mg., thiamine hydrochloride 0.3 mg., riboflavin 0.6 mg., calcium pantothenate 2.0 mg., choline chloride 150 mg., nicotinic acid 5 mg., biotin 0.02 mg., pyridoxine hydrochloride 0.4 mg., 2-methyl-1,4-naphthoquinone 0.05 mg., and α -tocopherol 0.3 mg. In addition each chick received weekly by dropper 1200 U. S. P. units of vitamin A and 120 A. O. A. C. units of vitamin D₃. After 3 days on the basal diet chicks within a 10 gm. weight range were divided into uniform groups of six and given the supplements shown in Table I. Streptomycin, streptothricin, and sulfasuxidine supplements were ground in a mortar with 10 gm. of the casein prior to inclusion in the diet. All experiments were terminated when the chicks were 4 weeks old and representative groups were chosen for bacteriological analysis of their cecal contents (8).

Results

The addition of 5000 units of streptothricin per 100 gm. of basal diet had no significant effect on growth or survival of the chicks, while levels of 10,000 units or more proved toxic. The addition of 500 γ per cent of folic acid proved ineffective in counteracting this toxicity. Liver eluates, whole liver substance, Wilson's liver fraction L singly or in combination with 500 γ per cent of folic acid were likewise found ineffective. Histamine hydrochloride at a level of 0.02 per cent was found non-toxic to the chicks.

Rather unexpected results were obtained with streptomycin in that increased growth was observed when this compound was fed together with adequate amounts of folic acid (compare Groups 11 and 12 with Group 4). Similarly, chicks receiving sulfasuxidine and 500 γ per cent of folic acid exhibited increased growth over those fed folic acid alone. A combination of sulfasuxidine and streptomycin in addition to the 500 γ per cent of folic acid produced a decreased growth when compared to either compound alone but an increased growth over the 500 γ per cent of folic acid control group. This observed growth stimulation by sulfasuxidine and streptomycin is interesting, since it suggests the inhibition of intestinal bacteria that are either producing toxic materials or are rendering certain dietary vitamins unavailable to the animal, an effect different than that usually encountered with sulfonamides. In this latter connection it is worth noting that Parsons (9) has induced a thiamine deficiency in humans by

TABLE I
Growth, Toxicity, and Bacterial Counts* in Chicks

Group No.	Supplement added per 100 gm. basal ration	No. of dead in 4 wks.†	Average weight at 4 wks.†	Total count	Coliform bacteria	<i>Escherichia coli</i>	<i>Enterococci</i>	<i>Lactobacilli</i>	Yeast
1	None	1	155	100	100	10	0.01	0.01	
2	Norit eluate‡ = 5% or 2% liver fraction L	0	220						
3	5% whole liver substance	0	245						
4	500 γ % folic acid	0	220	25,000	100	100	1000	25,000	
				25,000	2500	2500	1000	25,000	
5	5000 units streptothricin	0	150						
6	10,000 units streptothricin	5	50						
7	25,000 units streptothricin	6							
8	Streptothricin, 1000 units 1st wk., 5000 units 2nd wk., 10,000 units 3rd wk., 25,000 units 4th wk.	2	120	100	10		1	0.001	
9	5000 units streptothricin + 5% whole liver substance	0	240						
10	1% sulfasuxidine§ + 500 γ % folic acid	0	250	50,000	0.001	0.001	1	50,000	0.001
				7,500	0.001		<0.001	5,000	
11	10,000 units streptomycin + 500 γ % folic acid	0	280						
12	50,000 units streptomycin + 500 γ % folic acid	0	300	1,000	0.01	0.01	<0.001	1,000	0.001
				1,000	0.001	<0.001	<0.001	1,000	
13	10,000 units streptomycin + 500 γ % folic acid + 1% sulfasuxidine	0	240	1,000			<0.001	1,000	0.001
				1,000	0.001		0.1	1,000	

* Bacterial counts are given in terms of the number of bacteria per cc. of cecal contents $\times 10^4$.

† Weights for Groups 1 and 2 are based on twenty-four chicks.

‡ An alcohol-water-ammonia eluate of a charcoal adsorbate of liver fraction L. Expressed as equivalents per cent of the gm. of starting material per 100 gm. of ration.

§ Succinylsulfathiazole.

|| A concentrate containing 300 units per mg.

feeding large amounts of live yeast which supposedly is utilizing dietary thiamine for its own metabolism, thus leaving inadequate amounts available to the host. However, the possibility that these agents are acting systemically cannot be overlooked. Parenthetically, streptomycin did not appear to increase the biotin requirement of the chick above 20 γ per cent in contrast to the report of Emerson and Smith (10) who have induced biotin-like deficiency symptoms in rats by the oral administration of streptomycin. This different response indicates that the chick relies almost entirely on an external source of biotin and any synthesis occurring in the intestine is negligible. Alternatively, a partial synthesis of biotin might occur in the chick intestine but the organism responsible for this synthesis is not affected by streptomycin as in the case of the rat.

All of the sulfonamides failed to sterilize the intestinal tracts of the chicks and no significant decreases in the total count of the intestinal bacteria were observed (Table I). The number of animals used for the bacteriological studies was limited but the results substantiate the findings of Smith and Robinson (11) that orally administered streptomycin produces a marked reduction in the coliform bacteria of the feces. It is interesting to note that in the chicks receiving a low folic acid diet (Group 1) the coliform bacteria were the predominant microorganisms, while in the presence of folic acid (Group 4) the *Lactobacilli* predominate. Experiments are now in progress to verify this observation further.

SUMMARY

Streptothricin at levels of 5000 units per 100 gm. of purified diet is readily tolerated by the chick but when given at levels of 10,000 units or more is toxic. No toxicity was observed when streptomycin was fed at levels of 50,000 units per 100 gm. of diet. Sulfasuxidine and streptomycin singly or in combination lead to increased growth responses in chicks receiving our basal diet supplemented with adequate amounts of folic acid. Streptothricin, streptomycin, sulfasuxidine, or a combination of streptomycin and sulfasuxidine failed to sterilize the intestinal tract of chicks, but produced a marked reduction in the coliform bacteria of the cecal contents.

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DETERMINATION OF LIPASE ACTIVITY

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Heretofore most methods for the determination of lipase activity have employed emulsions of fatty acid esters in water (1). These emulsions have been stabilized by use of such agents as gum acacia. The disadvantages of such methods are that (1) the emulsions break up to a considerable and variable extent during the course of the incubation, and (2) the enzyme is presumed to be in the aqueous phase, whereas the substrate is only dispersed (not in solution) in the aqueous phase. Attempts to study the relation of substrate concentration to rate of hydrolysis are complicated by the fact that the substrate and enzyme are in different phases. The rate of hydrolysis in these cases is dependent partly on the degree of dispersion of the substrate rather than on its concentration.

Some of the commercially available esters¹ of sorbitan and fatty acids are completely soluble in water. Use of solutions of these esters permits one to study the effect of substrate concentration on enzyme activity and eliminates the complications which attend the use of emulsions. Gomori (2) has employed esters with the trade names Tween 40 and Tween 60¹ as substrates to detect, and to localize histologically, lipase activity in sections of tissue. This paper outlines a method of measuring quantitatively lipase activity in which an aqueous solution of a polyoxyalkylene derivative of sorbitan monolaurate is used as substrate.¹ For convenience this substrate will be designated hereafter by its trade name, Tween 20.

Apparatus—

Burette, 10 cc. It is convenient to employ a burette connected with a reservoir for NaOH. Both burette and reservoir should be fitted with soda lime tubes to protect the NaOH from atmospheric CO₂.

30 cc. glass-stoppered bottles (Pyrex) or 30 cc. heavy walled test-tubes with footed stirring rods 2 inches longer than the test-tubes.

¹ Tweenes and Spans are obtained from the Atlas Powder Company, Wilmington 99, Delaware. Tween 20, a clear yellow liquid, is a mixture of polyoxyalkylene derivatives of an ester of 1 mole of fatty acid per mole of sorbitan. Although the Tween contains more than one ester, the composition of different batches is so uniform that activities, as determined with three different lots, were identical. Tween and Span are registered United States trade marks.

*Reagents—**Syrupy phosphoric acid.* *$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$.**2-Methyl-2,4-pentanediol.²**Ether Mixture A*, 1 part of diethyl ether and 2 parts of petroleum ether.*Ether Mixture B*, 5 parts of diethyl ether and 1 part of petroleum ether.*Phosphate buffer*, 0.5 M, pH 6.8.*0.01 N NaOH solution.*

Solution of Tween 20,¹ 50 per cent (by volume) solution of the fatty acid-free Tween in water titrated to pH 6.8 with 18 N NaOH. This should be prepared fresh daily.

Removal of Free Fatty Acid from Tween—30 cc. of Tween 20¹ and 50 cc. of the ether Mixture A are placed in a 100 cc. glass-stoppered bottle; 0.25 cc. of H_3PO_4 is added. The mixture is shaken vigorously by hand for 15 seconds, then centrifuged (in the stoppered bottle). With the aid of a vacuum line or water vacuum pump the supernatant ether layer containing the fatty acid is removed through a capillary. Another 25 cc. of ether Mixture A are added and the shaking, centrifugation, and withdrawal of supernatant are repeated. The ether dissolved in the Tween layer is then removed by applying a partial vacuum. The Tween from which the free fatty acids have been removed is adjusted to pH 6.8 with NaOH, then decanted from the supernatant crystals of sodium phosphate.

Alternative Method of Removing Fatty Acids—30 cc. of Tween 20 and 0.25 cc. of syrupy phosphoric acid are placed in a Kutscher-Steudel (3) extractor and extracted for 4 hours with Mixture A. Nearly all of the fatty acid and a small amount of an almost colorless fraction of Tween are extracted by the ether mixture. The residual Tween is then neutralized with 18 N NaOH (about 0.20 cc.) and the dissolved ether is removed under reduced pressure.

Preparation of Lipase Solution—The lipase preparation should be freshly diluted with water so that 1 cc. will liberate between 0.01 and 0.09 mm of fatty acid per hour; 0.5 cc. of a 1 per cent suspension of commercial pancreatic lipase liberates about 0.06 mm per hour at 25°.

Procedure with Glass-Stoppered Bottles—3 cc. of 50 per cent solution of neutralized Tween 20, 0.5 cc. of phosphate buffer, and 0.5 cc. of lipase solution are mixed in a 30 cc. glass-stoppered Pyrex bottle and allowed to react for 1 hour at 25°. As a control, in a second bottle, another portion of the neutralized Tween 20 solution is set up simultaneously with buffer but without lipase. Lipase solution for the control is allowed to stand in a separate container, and added to the control solution later. At the

² 2-Methyl-2,4-pentanediol is obtained from the Commercial Solvents Corporation.

end of the reaction period, 1.6 gm. of solid $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and 15 cc. of the ether Mixture B are added to each. After mixing the solid phosphate with the solutions, 0.5 cc. of lipase solution is added to the control. Both bottles are stoppered and shaken vigorously for 30 seconds, then centrifuged. With the aid of suction and a fine capillary the supernatant aqueous solution of phosphate and the intermediate layer of Tween are removed from each bottle. By tilting the bottles all but a drop or two of Tween can be removed without loss of ether. The ether is then readily decanted into clean 25 cc. beakers. Each bottle is rinsed twice with 3 cc. portions of the ether mixture and the washings are added to the corresponding beakers.

A 0.5 cc. portion of 2-methyl-2,4-pentanediol is added to each beaker and the ether is evaporated by placing the beaker on a warm surface. The diol keeps the fatty acid in solution. 1 drop of thymol blue is added to each residue and the acid is titrated with 0.01 N NaOH. If ethyl alcohol is used to replace the diol, the color at the end-point is transient, owing presumably to oxidation of alcohol to acetic acid.

Alternative Procedure with Test-Tubes for Incubation and Extraction—Incubation and extraction can be conducted in test-tubes almost as readily as in glass-stoppered bottles. When test-tubes are employed, the procedure is exactly as outlined above except that the ether and aqueous phases are mixed by the rapid up and down motion of a footed stirring rod. The layers can be mixed thoroughly and easily without loss of fluid provided the foot of the rod is kept below the upper surface of the ether phase during the mixing. After 4 minutes mixing the rod is removed and the tube is capped and centrifuged (4000 R.P.M.) for 5 minutes.

Calculation—The milliequivalents of acid split off per hour = $(D - C) / (100 \times T)$ where D = the cc. of 0.01 N NaOH required to titrate the acid extracted from the digest, C = the cc. of NaOH required by the control, and T = the duration of the incubation in hours.

Discussion of Method

A polyoxyalkylene derivative of a sorbitan lauric acid ester was selected as a substrate in preference to an ester of other acids, because it is completely soluble in water. Furthermore, lauric acid esters are said to be split by pancreatic lipase more readily than esters of other acids ((1), p. 70).³ Although triglycerides are said to be more rapidly hydrolyzed than di- or monoglycerides, the polyoxyalkylene sorbitan monolaurate was employed because of its complete solubility in water.

Tween 20, in the absence of water, is soluble in diethyl ether, but in-

³ Approximately 60 per cent of the fatty acid in Tween 20 is lauric acid.

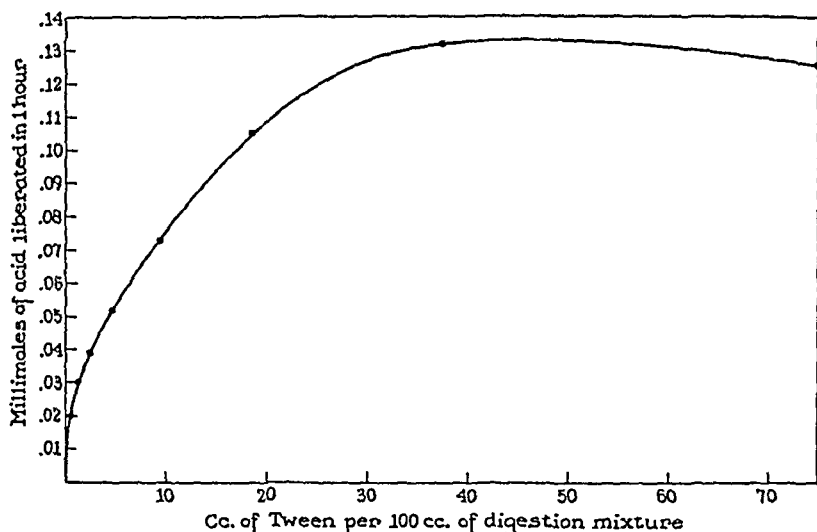


FIG. 1. Curve showing the relation of enzyme activity at 25° and pH 6.8 to the concentration of Tween 20 in the reaction mixtures; 0.5 cc. of 2 per cent pancreatic lipase was added to 3.5 cc. of the mixture of Tween and buffer.

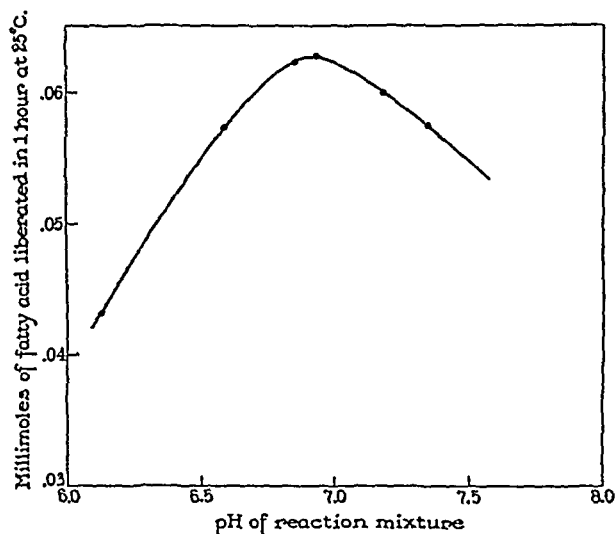


FIG. 2. Curve showing the relation of enzyme activity at 25° to pH. 0.5 cc. aliquots of a 1 per cent pancreatic lipase were added to mixtures of 0.5 cc. of buffer and 3 cc. of 50 per cent Tween 20.

soluble in petroleum ether. If higher concentrations of petroleum ether are employed in place of Mixture A, the free fatty acid would be removed less completely. Higher concentrations of ethyl ether would result in

the extraction of too much Tween. In the presence of water the Tween is much less soluble in ethyl ether. Hence a larger ratio of ethyl ether is

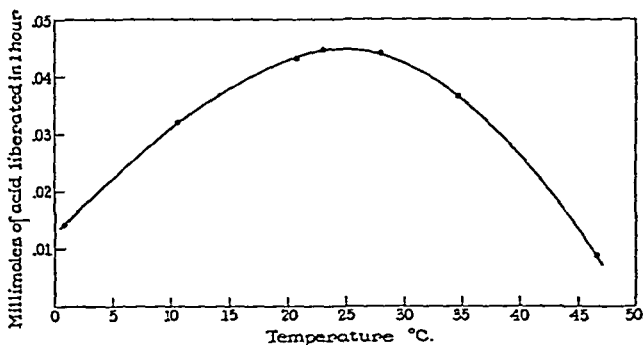


FIG. 3. Curve showing the effect of temperature on the amount of acid liberated from Tween by pancreatic lipase. Except for temperature the conditions were as outlined in the procedure for determination of lipase activity.

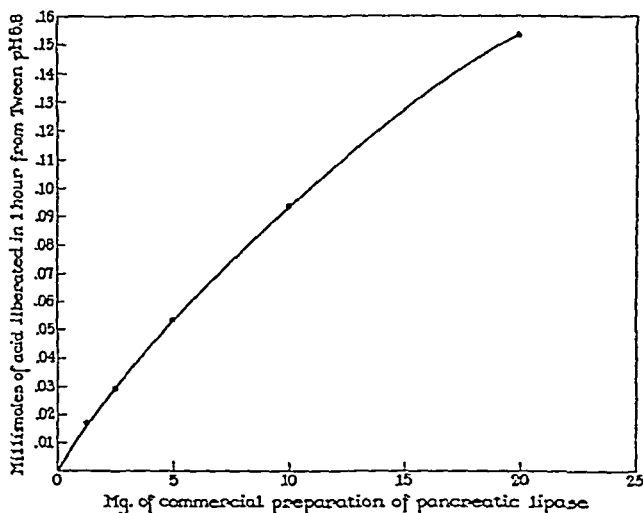


FIG. 4. Curve showing the relation of the amount of enzyme present to the amount of acid liberated from the substrate in 1 hour. The substrate concentration is 37.5 per cent.

used in Mixture B. If the ratio of petroleum ether is increased much over that recommended for Mixture B, difficulty will be experienced in ob-

taining clear supernatant solutions. It is important to add sufficient $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ to saturate the aqueous phase; otherwise the three phases (ether, Tween, and phosphate solution) will not separate sharply.

Results

Fig. 1 indicates how the amount of fatty acid liberated in the presence of a constant amount of pancreatic lipase varies with the concentration of substrate at 25° and pH 6.8.

Fig. 2 shows how the pH of the mixture (with phosphate buffer) affects the rate of hydrolysis by pancreatic lipase when the substrate concentration is constant (at 37.5 per cent).

Fig. 3 illustrates the effect of temperature on the rate of reaction of pancreatic lipase in a 37.5 per cent solution of Tween 20 at pH 6.8.

Fig. 4 indicates the amount of hydrolysis obtained with varying amounts of enzyme at pH 6.8.

The data presented in Figs. 1 to 4 indicate the application of the method to the determination of lipase activity in preparations of defatted pancreas.

Discussion of Results

The fact that the rate of action of lipase increases as the substrate concentration is increased up to 37.5 per cent is not surprising in view of the fact that the action of the enzyme is so readily reversible and that equilibrium with oleic acid is reached when about 50 per cent (in the case of pancreatic lipase) (4) or 40 per cent (in the case of *Ricinus* lipase) (5) of the fatty acid is combined with glycerol ((1), p. 68).

The author is indebted to Dr. B. Davis for outlining his somewhat similar method for the determination of fatty acid.

SUMMARY

1. A simple method for the determination of lipase activity is presented. A substrate, Tween 20, which is completely soluble in water is employed. A reaction period of 1 hour is adequate.

2. The optimum pH, temperature, and concentration of substrate for the system have been demonstrated.

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A DIETARY FACTOR ESSENTIAL FOR GUINEA PIGS*

IX. TISSUE METABOLISM DURING THE DEFICIENCY

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Relatively few data are available demonstrating the effect of vitamin deficiencies upon the respiratory mechanisms of animal tissues. Chevallier and Roux (1) investigated the effect of added vitamin A to liver slices of vitamin A avitaminotic rats and guinea pigs and compared this effect with that obtained when vitamin A was added to liver slices of these animals receiving an adequate amount of vitamin A. No difference in the oxygen uptake was noted. Shibata (2) reported a slight increase in the oxygen consumption of the liver from rats during avitaminosis A. The kidneys and spleen of the same animals had a lower respiratory rate than those of normal animals. Ruddy (3) concluded from her observations that vitamin A is not important in the respiration of liver tissues. Stotz, Harrer, Schultze, and King (4) observed a marked increase in the oxygen uptake of liver slices from scorbutic guinea pigs, as compared to that of normal animals. Shibata (2), on the contrary, reported no changes in the liver respiration and only a slight inhibition of the respiratory rate for the kidney of vitamin C-deficient guinea pigs. The effects of a thiamine deficiency upon the respiratory rate of rat tissues have been described by several investigators. Goldschmidt and Lewin (5) reported a decrease in the oxygen uptake of liver slices of vitamin B₁ avitaminotic rats. Under similar conditions, according to Hastings, Muus, and Bessey (6) and Muus, Weiss, and Hastings (7) the ventricular muscle tissue of the rat heart retains its normal rate of respiration, whereas the rate of the oxygen consumption of auricular tissue is doubled. Addition of thiamine to the substrate decreased the oxygen consumption of the avitaminotic liver, while it markedly increased that of normal liver. Respiration of the diaphragm is accelerated during riboflavin deficiency (6).

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† Based in part on a thesis submitted by one of the authors (D. H. S.) in partial fulfillment of the requirements for the degree of Master of Arts, Oregon State College. A part of this study was presented before the Division of Biological Chemistry at the 109th meeting of the American Chemical Society at Atlantic City, April 8-12, 1946.

Muscular dystrophy is generally accompanied by an increased oxygen consumption in the muscle (8, 9). Kaunitz and Pappenheimer (10) observed changes in the *in vitro* oxygen consumption of rat and chick muscles during vitamin E deficiency. The oxygen uptake of both the rat and chick muscles increased, while that of the rat liver remained unchanged. Houchin (11) and Houchin and Mattill (12) have confirmed these results with experiments on the muscle tissue from hamsters and rabbits deficient in vitamin E. These authors suggest a direct action of vitamin E upon the succinoxidase system.

Guinea pigs raised on a diet deficient in the antistiffness factor (13) develop a characteristic syndrome (14). The first outward sign of the deficiency is the development of a stiffness at the wrist joint. In the advanced stages of the deficiency the muscles are found to be extremely atrophied. The deficiency of this factor also results in an abnormal distribution of the acid-soluble phosphorus in the liver, kidneys (15), and muscle (16) of the experimental animals. The fractions representing adenosine tri- and diphosphate in these tissues are lowered far below normal. An abnormal aerobic and anaerobic glycolysis of these tissues could be expected as a result of the changes in the phosphorus distribution mentioned above. A significant derangement of both mechanisms was found in all tissues.

EXPERIMENTAL

Method

Guinea pigs, weighing from 300 to 350 gm., were segregated as to sex and housed in clean cages on autoclaved straw. The animals were fed *ad libitum* the deficient diet as described by van Wagendonk (15). This diet consisted of skim milk powder to which adequate amounts of water, minerals, and the known vitamins were added. The average daily food intake was 280 gm. The animals on the deficient diet gained as regularly in weight as the animals receiving a stock diet (consisting of rolled barley and greens), the average weekly gain in weight being 20 to 30 gm. in the 1st year. In the later stages of the deficiency a cessation of growth and loss of weight occurred. The experimental animals did not show signs of any deficiency diseases, except a constantly increasing stiffness at the wrist joint.

Preparation of Tissue Slices—The animals were sacrificed at various intervals by decapitation. The tissues (liver, kidney, and muscle) were excised as rapidly as possible and transferred to a cold moist chamber for slicing (17). Liver slices of desired thickness (± 0.5 mm.) were prepared with the Martin slicer (18). Slices of the kidney cortex were cut with a modified Terry microtome (19). The muscle strips were teased out of the hind legs

according to the method of Shorr (20). The tissue preparations were immediately weighed and transferred to the conical flasks of the Warburg respirometer, or to small drying tubes, and dried to constant weight at 105°.

Determination of Oxygen Consumption—The oxygen consumption was determined with the Warburg respirometer at 37.0°; a phosphate-Ringer's solution modified according to Dickens and Greville (21) was used as substrate and purified oxygen as the gas phase. 0.2 cc. of 10 per cent KOH solution and a strip of filter paper were placed in the center well to absorb the carbon dioxide formed.

Determination of Anaerobic Glycolysis—The anaerobic glycolysis was also determined with the Warburg apparatus at 37.0°, with the substrate described by Krebs and Henseleit (22). The alkali solution and the filter paper strip were omitted from the center well. The gas phase was a mixture of 95 per cent N₂ and 5 per cent CO₂ purified in a unit modeled after that described by Savage and Ordal (23). Readings for both determinations were taken every 10 minutes for 90 minutes after a 15 minute equilibration period. At least one duplicate determination was made on the same tissue and in a number of cases triplicate determinations were carried out.

The oxygen consumption and the anaerobic glycolysis are expressed respectively per mg. of initial dry weight per hour as c.mm. of O₂ at normal temperature and pressure (Q_{O_2}) and c.mm. of CO₂ ($Q_{CO_2}^{N_2}$), with the wet to dry ratios of the different tissues (see Table I). The initial dry weight is used in preference to the final dry weight for the calculations of Q_{O_2} and $Q_{CO_2}^{N_2}$ because of the variability of the latter value resulting from imbibition of water (24) and the probability of incomplete recovery of tissue from the vessel at the end of the determination (25).

Glycogen was determined by a direct colorimetric method (26). All data were analyzed statistically according to the methods of Fisher (27).

Results

For the series of animals used no significant difference could be noted between the initial wet to dry ratio of the liver, muscle, and kidney cortex tissues of either the animals receiving the stock diet or those raised on the deficient diet (Table I). This ratio was also constant for all deficient animals regardless of the length of the period during which the skim milk diet was received.

In Table II are represented the values obtained for the Q_{O_2} and $Q_{CO_2}^{N_2}$ for liver tissue of normal and deficient animals. The oxygen consumption increased, while the anaerobic glycolysis decreased during the deficiency. The Q_{O_2} and the $Q_{CO_2}^{N_2}$ attained the reported levels of respectively 4.08 and 1.69 after the animals had consumed the deficient diet for 1 week. No

significant variations were found during the period of the investigation. The liver slices of animals which had received the diet for 77 weeks had a $Q_{O_2} = 4.12$ and a $Q_{CO_2}^{N_2} = 1.95$ (average of the values obtained from four guinea pigs). The value for the Q_{O_2} for normal guinea pig liver was found to be lower than generally reported (1, 4, 9, 28, 29). This can be explained by the fact that the methods used by other authors have not been adequately controlled (30).¹

TABLE I,

Water Content of Liver, Muscle, and Kidney Tissues of Guinea Pigs Raised on Stock Diet and on Diet Deficient in Antistiffness Factor

Tissue samples were taken immediately after death and exsanguination, and dried to constant weight at 105°. All determinations were made in duplicate. The Q_{O_2} and $Q_{CO_2}^{N_2}$ on a wet weight basis may be converted to Q_{O_2} and $Q_{CO_2}^{N_2}$ on a dry weight basis by multiplication by the wet to dry ratio.

Tissue	Diet	No. of determinations	Mean per cent water	Standard deviation	Standard error	Mean wet to dry ratio
Liver	Stock	17	73.50	0.23	0.055	3.77
	Skim milk	23	72.15	0.23	0.058	3.64
Muscle	Stock	16	79.20	0.49	0.123	4.80
	Skim milk	24	77.90	0.49	0.099	4.51
Kidney	Stock	15	78.80	0.34	0.088	4.70
	Skim milk	21	79.00	0.35	0.076	4.73

TABLE II

Oxygen Consumption and Anaerobic Glycolysis of Liver Slices of Normal and Deficient Guinea Pigs

Diet	Q_{O_2} (20 determinations)	$Q_{CO_2}^{N_2}$	
	Mean \pm s.e.	No. of determinations	Mean \pm s.e.
Stock	2.83 \pm 0.18 (1.83-3.98)	18	3.04 \pm 0.20 (1.95-4.10)
Deficient	4.08 \pm 0.20 (3.08-5.07)	19	1.69 \pm 0.17 (0.69-2.80)

The figures in parentheses indicate the range of observed values.

Values for the two metabolic processes in deficient kidney cortex and muscle tissue are found in Table III. At first sight the situation in the

¹ It is interesting to note that the diet used by Stotz *et al.* (4) contained 50 per cent heated skim milk powder. Since the animals used in our investigations were adequately supplied with ascorbic acid, it might be suggested that the similar changes in the Q_{O_2} observed by these authors were not due to a vitamin C deficiency but to a deficiency of the antistiffness factor.

TABLE III
Oxygen Consumption and Anaerobic Glycolysis of Kidney Cortex Slices and Muscle Strips of Normal and Deficient Guinea Pigs

Diet	Time on diet	Kidney cortex					Muscle				
		Q_{O_2}		Total activity,* mean	Q_{CO_2}		Q_{O_2}	No. of deter- mina- tions	Mean \pm s.e.		
		No. of deter- mina- tions	Mean \pm s.e.		No. of deter- mina- tions	Mean \pm s.e.					
Stock	13-20 wks.	12	10.90 \pm 0.30 (8.00-14.70)†	3.38	0	4.04 \pm 0.44 (2.52-7.38)	1.53	12	2.73 \pm 0.15 (1.77-3.49)	0	4.70 \pm 0.38 (2.43-5.91)
Skim milk	2-3	5	10.40 \pm 0.40 (8.80-11.70)	3.21				5	2.50 \pm 0.12 (1.72-3.31)		
"	9-10	3	11.40 \pm 0.53 (10.90-12.30)	3.52				3	2.52 \pm 0.00 (2.31-2.70)		
"	13-14	3	8.30 \pm 0.30 (7.60-9.10)	3.08				4	2.60 \pm 0.00 (2.30-2.81)		
"	20-24	6			8	5.40 \pm 0.52 (3.08-6.55)	2.00			8	4.73 \pm 0.40 (3.30-6.53)
"	30-35	4	8.50 \pm 0.33 (8.13-9.45)	3.12	3	5.40 \pm 0.06 (5.23-5.47)	2.00	3	2.26 \pm 0.05 (2.22-2.33)		
"	66-77	4	6.70 \pm 0.55 (5.10-7.90)	3.00	3	5.11 \pm 0.25 (4.48-5.58)	2.50	3	1.95 \pm 0.25 (1.51-2.51)	3	4.25 \pm 0.47 (3.03-4.78)

* Total activity is Q_{O_2} (or Q_{CO_2}) times per cent kidney in the guinea pig.

† The figures in parentheses indicate the range.

TABLE IV
Influence of Administration of Antistiffness Factor to Guinea Pigs Deficient in This Factor on Oxygen Consumption and Anaerobic Glycolysis of Liver, Kidney Cortex, and Muscle

Diet (35 wks.)	Liver				Kidney cortex				Muscle			
	Q_{O_2}		$Q_{CO_2}^{N_2}$		Q_{O_2}		$Q_{CO_2}^{N_2}$		Q_{O_2}		$Q_{CO_2}^{N_2}$	
	No. of deter- minations	Mean \pm s.e.	No. of deter- minations	Mean \pm s.e.	No. of deter- minations	Mean \pm s.e.	No. of deter- minations	Mean \pm s.e.	No. of deter- minations	Mean \pm s.e.	No. of deter- minations	Mean \pm s.e.
Deficient	3	4.70 \pm 0.52 (3.78-5.92)	3	1.69 \pm 0.110 (1.48-1.96)	4	8.50 \pm 0.33 (8.13-9.45)	3	5.40 \pm 0.06 (5.23-5.46)	3	2.26 \pm 0.05 (2.19-2.31)	3	5.00 \pm 0.03 (4.21-5.38)
" and antistiffness factor	6	2.78 \pm 0.15 (2.33-3.36)	6	2.18 \pm 0.39 (1.00-4.10)	7	7.63 \pm 0.42 (6.45-9.50)	7	6.16 \pm 0.44 (4.61-8.36)	6	2.19 \pm 0.12 (1.82-2.56)	6	4.88 \pm 0.55 (2.88-6.42)

The figures in parentheses indicate the range.

kidney and the muscle seems to be the reverse from that reported for the liver. A decrease of the Q_{O_2} becomes apparent only after the deficiency has progressed beyond the 10th week. Even then there is a slight gradual decrease to a value of 6.70 which is attained after more than a year. Apparently no significant deviations from the normal values for the $Q_{CO_2}^N$ are found. However, the kidneys of the deficient guinea pig are from 1.5 to 2

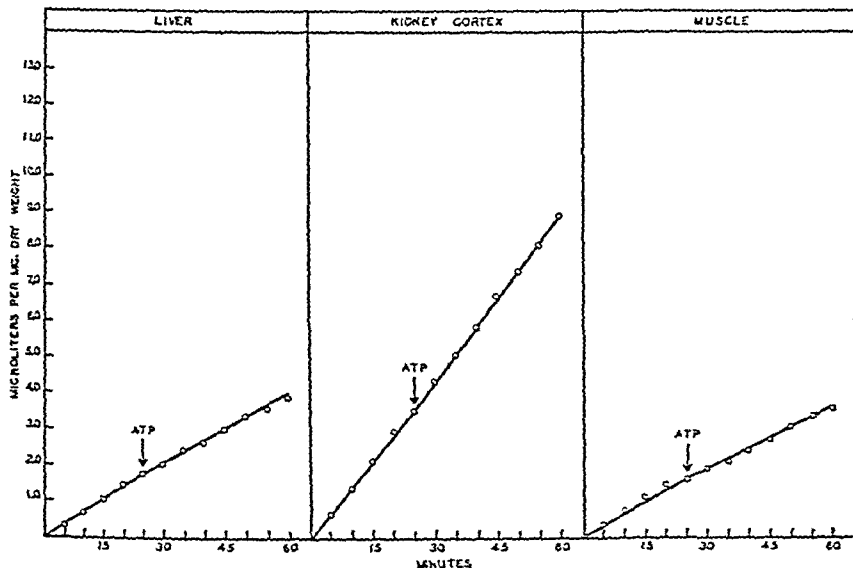


FIG. 1. The oxygen consumption of tissues from deficient guinea pigs (36 weeks deficient) before and after addition of adenosine triphosphate (ATP). The side cup contained ATP dissolved in phosphate-Ringer's buffer according to the procedure of Dickens and Greville (0.5 cc., 0.012 M) adjusted to a pH of 7.4. The main compartment contained 1.5 cc. of phosphate-Ringer's solution. After the contents of the two cups (indicated by the arrows) were mixed, the final concentration of ATP was 0.003 M. The points on the curves represent the averages from four different animals (triplicate determinations per tissue).

times larger than those of the normal animal of the same age. Since this is due to tissue proliferation (there is no difference in the wet to dry ratio, as reported in Table I), the value of these processes in the intact organ would be greater. It can be seen from Table III that the Q_{O_2} (total activity) would remain constant; the $Q_{CO_2}^N$ (total activity) on the other hand would increase. Some objections could be raised against this extrapolation, since we only determined the rates of respiration and of anaerobic

glycolysis in the kidney cortex, while the proliferation of the kidneys is not solely due to proliferation of this tissue. However, the cortex is certainly the most actively metabolizing part of the kidney and, since this tissue, too, has proliferated, a calculation on the above basis would seem justified.

The respiratory rate of muscle tissue decreases significantly only after

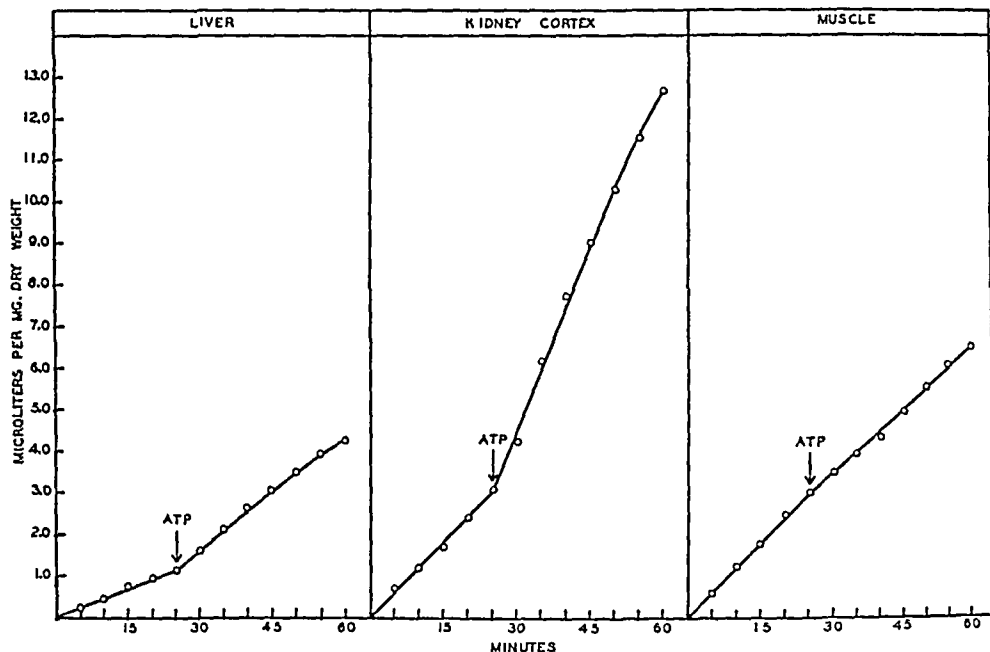


FIG. 2. Anaerobic glycolysis of tissues from deficient guinea pigs (36 weeks deficient) before and after addition of adenosine triphosphate (ATP). The side cup contained ATP dissolved in bicarbonate-Ringer's solution, according to the method of Krebs and Henseleit (0.5 cc., 0.018 M), saturated with CO_2 and carefully adjusted to pH 7.4. A careful adjustment of the pH of the solutions in the two compartments is essential. If the pH of the ATP solution is 0.1 unit lower than that of the substrate, mixing will cause a change of pressure corresponding to 20 mm. of Brodie's fluid, due to liberation of CO_2 . The main compartment contained 2.5 cc. of bicarbonate-Ringer's solution treated similarly. After the contents of the two cups were mixed (indicated by the arrows), the final concentration of ATP was 0.003 M. The points on the curves represent the averages from six animals (triplicate determinations per tissue).

the 13th to 14th week, therefore coinciding with the decrease in the concentrations of creatine phosphate and adenosine tri- and diphosphate (16). A slight but not significant decrease in the anaerobic glycolysis is found only after the animals had received the deficient diet for a period of over a year.

If the antistiffness factor is a regulating factor in the metabolic processes,

the influence of the administration of this compound to the deficient animal would be reflected in changes in the Q_{O_2} and $Q_{CO_2}^{st}$ of the tissues under investigation. Experiments to test this inference are reported in Table IV. During the last 5 days of the experiment 2 γ (1000 units) of the factor were administered to animals which had received the deficient diet for 35 weeks. Both the respiratory rate and the anaerobic glycolysis of the liver showed a distinct tendency to return to normal values, although the normal rates are not completely attained. No significant change could be found in the respiratory rate of the kidney cortex, although a small decrease was notice-

TABLE V
Effect of Varying Dosages of Antistiffness Factor on Liver Glycogen of Deficient Guinea Pigs

Diet	Time on diet	No. of determinations	Days of treatment, 2 γ per day	Glycogen, wet weight
	hrs.			per cent
Deficient	3-72	16		0.55 ± 0.05 (0.20-0.93)*
" on cure	10	2	1	0.08
" " "	10	2	2	0.22
" " "	10	2	3	0.11
" " "	10	3	4	0.24
" " "	10	2	5	0.15
" " "	10	3	6	0.18
" " "	10	2		0.93
" " "	10	3		2.38
" " "	10	2		5.94
" " "	10	3		2.83
" " "	10	3		4.70
" " "	10	3		1.93
" " "	10	3		2.91
" " "	10	3		3.82
Stock		10		2.93 ± 0.29 (1.51-4.36)*

* Means \pm s.e. (values in parentheses indicate the range).

able. Contrary to the expectation the rate of anaerobic glycolysis showed an increase rather than a decrease. No significant influence of the administration of the antistiffness factor upon the two metabolic processes in the muscle was apparent.

A decrease of the easily hydrolyzable phosphorus fractions in liver, kidney, and muscle (16) might well be the cause for the deranged metabolic processes under investigation, since it is a well established fact that the adenylic acid system is to a large extent responsible for a smooth functioning of the energy-producing processes of respiration and anaerobic glycolysis. The antistiffness factor would then exert its regulatory effect indirectly

through its influence upon the functioning of the adenylic acid system. If this were the case, addition of adenosine triphosphate (ATP) to the substrate should restore both aerobic and anaerobic reactions to the rate found in normal tissues. It was of considerable importance to test this hypothesis. The results of pertinent experiments are represented in Figs. 1 and 2. The results of four determinations of the respiratory rate of deficient liver, kidney cortex, and muscle tissue before and after addition of ATP to the substrate (final concentration 0.003 M) are given in Fig. 1. The *in vitro* addition of ATP causes a slight increase in the Q_{O_2} of kidney cortex slices. No effect of the addition of ATP on the Q_{O_2} of liver and muscle tissue could be demonstrated.

However, addition of ATP had a striking effect on the anaerobic process in all three tissues (Fig. 2). An increase in the anaerobic glycolysis rate from 3.0 to 5.6 is found in the liver tissue. A similar and even larger increase (from 7.3 to 18.6) is found in the kidney cortex slice. A slight decrease in the anaerobic glycolysis of muscle tissue results when ATP is added to the metabolizing tissue.

A high respiratory rate might indicate that glycogen is oxidized directly, instead of being degraded via the normally occurring phosphorylating oxidation-reduction cycles. A low concentration of glycogen might then be expected in the deficient animal. This was actually found, as can be seen in Table V. This is also in accordance with the fact that a high inorganic phosphate in the liver is usually accompanied by a low glycogen concentration. In previous investigations (15) a high inorganic phosphate concentration was found to exist during the whole course of the deficiency. Administration of the antistiffness factor (1000 units per day = 2 γ) resulted in a gradual return to normal values.

DISCUSSION

The complete interpretation of the results obtained in this investigation is as yet difficult. It becomes more and more evident that the antistiffness factor plays an important rôle in the energy metabolism through its influence on the adenylic acid system. The antistiffness factor might either restrain the activity of the adenosinetriphosphatase and the adenosinepyrophosphatase, or act as a prosthetic group which promotes the rephosphorylation of adenylic acid. In the first case a deficiency of the factor would promote the rapid breakdown of any adenosine triphosphate formed during the metabolic cycles, resulting in an increase of inorganic phosphate and adenylic acid (or further breakdown products) in the tissues. The metabolic cycles would not be slowed down, but would proceed at a higher rate, since adenylic acid would become available in large amounts to act as an acceptor for the energy-rich phosphates formed during both the anaerobic

robic glycolysis and the following tricarboxylic acid and cytochrome cycles. The rapid dephosphorylation of the formed adenosine triphosphate would, however, seriously interfere with the energy metabolism, because the conversion of energy-rich phosphate compounds into inorganic phosphorus would mean a loss of the stored energy which would then not be available for other reactions requiring the expenditure of energy.

If, however, the antistiffness factor would act as a prosthetic group for the enzyme responsible for the phosphorylation of adenylic acid, a more direct interference with the normal metabolic cycles would result. The phosphorylation of adenylic acid would then be blocked in the deficient tissue, and the adenylic acid system would not be able to act as a phosphate acceptor for the energy-rich phosphate groups formed during the metabolic cycles. Especially the anaerobic glycolysis would then be slowed down.

The results obtained in the experiments with liver tissue point in this direction. In the deficient animal there is a definite shift towards the oxidative processes. The anaerobic glycolysis is decreased considerably, indicating that the normal pathway of carbohydrate utilization leading towards the hub of pyruvic acid is being by-passed. Warburg and co-workers (31, 32) have shown that animal tissues can oxidize glucose-6-phosphate directly to phosphogluconic acid and there is increasing evidence from other laboratories that a series of reactions, possibly starting with the direct oxidation of glucose-6-phosphate, is intervening early in the glycolysis of carbohydrate (33, 34). Since a direct oxidation of glucose to phosphogluconic acid will of necessity provide less energy to the animal organism than the normal pathway of carbohydrate utilization, a by-passing of the fermentation reactions will lead to a more extensive use of the glycogen formed in the liver. A small amount of liver glycogen would result from the heavy demands. It was actually found that the amount of glycogen in the liver of deficient animals is decreased to about 1 to 5 per cent of that present in normal animals. The addition of adenosine triphosphate results in both cases in a return to normal conditions of the aerobic and anaerobic metabolic processes, thereby lending support to the proposed theory of the action of the antistiffness factor.

The results obtained in the experiments with kidney slices are not so clear. The respiratory rate is decreasing slowly, while the anaerobic glycolysis rate remains constant, when the rates of both processes are calculated on a dry weight basis. If, however, the rates are expressed in relation to the whole organ, the Q_{O_2} remains constant, while the $Q_{CO_2}^{N_2}$ attains a higher value than in the normal animal. Administration of the antistiffness factor to the deficient animal has no significant effect on the respiratory rate, while the anaerobic glycolysis rate is but slightly increased. The

great increase in the anaerobic glycolysis after the addition of adenosine triphosphate to the substrate is surprising. It might be explained on the basis that, although the available supply of adenosine triphosphate in the deficient kidney is low, it apparently is sufficient to maintain the full functioning of the metabolic cycles. Addition of adenosine triphosphate, or relieving the blocking of the phosphorylation reaction through the administration of the antistiffness factor, will in the beginning merely result in an acceleration of the metabolic reactions in which adenosine triphosphate is a coreactant. A somewhat similar situation prevails in the muscle. Addition of adenosine triphosphate to the isolated tissue produces no larger stimulation than does the *in vivo* administration of the antistiffness factor.

There remains, however, the possibility that the administration of the antistiffness factor during the short period of 5 days does not result in a complete return to normal conditions which would be a prerequisite for the normal functioning of the metabolic cycles. Evidence for this time lag was previously found in the slow return to normal values of the calcium concentration in the muscle (35) and the slow recovery of the abnormal protein distribution in the plasma (36). The addition of adenosine triphosphate to the isolated tissue might have a more immediate effect.

It would be of great interest to know where the exact point of interference with the normal metabolic cycles is located in the deficient animal. Attempts towards the solution of this question will form the subject of following publications.

SUMMARY

A deficiency of the antistiffness factor results in a derangement of the respiratory rate and the rate of anaerobic glycolysis in the liver, kidney cortex, and muscle. An increase in the Q_{O_2} and a decrease in the $Q_{CO_2}^{N_1}$ in the liver caused by the deficiency of this factor are reversible by the *in vivo* administration of the antistiffness factor or by the *in vitro* addition of adenosine triphosphate to the metabolizing tissue. These changes in the liver metabolism are correlated with a large decrease in the glycogen storage in the liver. A decrease of the Q_{O_2} and no changes in the $Q_{CO_2}^{N_1}$ could be demonstrated in the deficient kidney cortex and muscle. *In vivo* administration of the antistiffness factor did not materially alter the trend of the investigated processes during the time limit of the curative process. However, *in vitro* addition of adenosine triphosphate to the anaerobically metabolizing tissues did result in a sharp increase in the rate of the anaerobic glycolysis in the kidney. The implications of these changes are discussed.

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EXTRACTION AND PURIFICATION OF STREPTOMYCIN, WITH A NOTE ON STREPTOTHRICIN

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The early clinical studies on streptomycin have indicated the necessity of preparing larger quantities of purified material for extended tests in cases requiring high dosage. The purpose of this paper is to report an extraction procedure developed in this laboratory for the preparation on a pilot laboratory scale of a product which meets clinical standards with respect to potency, purity, and freedom from toxic contaminants. The line product obtained by this process has been purified further by chromatographic separation to yield fractions of chemical purity suitable for degradation studies directed toward determination of structure.

Several methods have appeared in the literature for the small scale preparation of streptomycin salts, ranging from crude material to crystalline products of analytical purity (1-7). In practically all methods a carbon adsorption step has been employed and at least one laboratory (7) has reported acetone precipitation of a methanolic concentrate. However, the carbon-acetone method here described offers certain advantages for use with pilot laboratory equipment. These advantages include simplicity of operation with only a single organic solvent under conditions which stabilize the streptomycin during the extraction period. Recovery of the acetone is accomplished after its separation from the active precipitate. The period of operation is short and streptomycin sulfate may be obtained in a single fraction representing an over-all yield of approximately 60 per cent at a purity of 400 to 600 units per mg.

In preliminary experiments, fermentation harvests and solutions of crude streptomycin were fractionally precipitated with acetone at pH 2 to 8. It was found that streptomycin hydrochloride would remain in solution at pH 2 with acetone concentrations up to 70 per cent and, after filtration from insoluble material, the activity could be precipitated either by increasing the acetone concentration to 90 per cent or by adding NaOH to pH 8. Because of its lesser solubility the sulfate could be precipitated with 70 per cent acetone at pH 2.5. By applying these solubility principles to a carbon-adsorbed streptomycin prepared as described by Carter *et al.* (3), a simplified method was developed for extracting streptomycin salts with improved purity and yields.

The method consists essentially of clarifying and decolorizing the fermentation harvests at a low pH; adsorbing the activity on carbon from the clarified, decolorized beer at neutral pH; eluting the activity from carbon with acidified aqueous acetone; and precipitating the activity from the eluate by increasing the acetone concentration and adjusting the pH. Either the hydrochloride or sulfate can be isolated, depending upon the acid used in elution. The method has also been applied to cultures of *Streptomyces lavendulae* (2) to yield streptothricin sulfate.

EXPERIMENTAL

Assay and Standards—The method of Loo *et al.* (8) was used and the activities are expressed in units. By exchange of standards with Dr. S. A. Waksman it was possible to correlate our unit with his *Escherichia coli* unit (1, 9). The tentative standard recently issued by the Food and Drug Administration is labeled in terms of micrograms of streptomycin base but was not available at the time this work was done. A comparison of standards carried out in this laboratory by Dr. R. L. Emerson indicates that 1 unit is equivalent to 0.97 γ of streptomycin base. Thus, the "unit" values used throughout this paper should be reduced by 3 per cent in order to express them in terms of the tentative "microgram" of the Food and Drug Administration.

Preparation of Streptomycin Sulfate—Submerged cultures of *Streptomyces griseus* harvested at a potency of 50 to 170 units per ml. were used as starting material. The fermentation was carried out in iron tanks with a medium of the following composition: dextrose (cerelose) 10 gm., Curbay B-G (soluble products from butanol fermentation, United States Industrial Chemicals, Inc.) 1 gm., sodium chloride 5 gm., basic potassium phosphate 1 gm., ammonium sulfate 2.5 gm., magnesium sulfate (7H₂O) 0.25 gm., calcium carbonate (sterilized separately) 3.5 gm., tap water to make 1 liter. The culture medium was seeded with a 5 per cent volume of a 48 hour vegetative inoculum. The quantities of fermentation liquor extracted in individual runs varied from 190 to 450 liters (see Table I). For convenience, the method is described for 100 liters of fermentation harvest.

100 liters assaying 120 units per ml. (total 12 million units) were acidified with H₂SO₄ to pH 2 and stirred for 30 minutes with 250 gm. of nuchar C-190-N. The mixture was filtered on a filter press with the aid of 2000 gm. of Celite 545 and the cake washed with 10 liters of water. The combined filtrate and washings were neutralized with NaOH to pH 7 and stirred with 1000 gm. of Darco G-60 for 30 minutes. The carbon containing the streptomycin was collected on a filter press with the aid of 500 gm. of Celite and washed twice with 10 liters of water. The streptomycin was eluted from the carbon by extracting three times with 3.5 liters of 5 to 10 per cent

acetone, acidified, and maintained at pH 2.5 with H_2SO_4 . The combined eluates were stirred with 3 volumes (28.5 liters) of acetone which precipitated the streptomycin sulfate. After being chilled overnight at 4° , the precipitate was collected by decanting and filtration. The precipitate was suspended in 250 ml. of pyrogen-free distilled water and inactive insoluble material was removed by filtration. The filtrate was adjusted to pH 7.0 with NaOH and additional insoluble material was removed by filtration. The clarified solution of streptomycin sulfate (volume 250 ml.) was frozen and dried *in vacuo* at 200 to 600 μ . The product was an off white powder weighing 12 gm. and assaying 600 units per mg., representing an over-all recovery of 7.2 million units or 60 per cent. The results for six pilot laboratory lots with the above fermentation medium are given in Table I.

TABLE I
Streptomycin Sulfate by Carbon-Acetone Process

Lot No.	Fermentation harvest		Recovery of streptomycin sulfate			
	Volume	Units	Weight	Potency	Units	Yield
	liters	millions	gm.	units per mg.	millions	per cent
141	260	45.0	35.7	632	22.6	50
153	195	24.6	24.2	488	11.8	48
156	260	13.8	19.0	530	10.1	73
158	190	23.2	28.2	660	18.0	78
159	318	42.6	47.9	551	26.4	62
167	450	68.0	69.0	650	44.9	66
168*	215	23.4	32.5	400	13.0	55

* Peptone-meat extract medium used in Lot 166; Curbay-salts medium used in the other lots.

Since in much of the previously reported work on streptomycin Waksman's peptone-meat extract medium has been employed, it was believed desirable to test the extraction method on this type of fermentation liquor. Lot 166 was fermented with a peptone-meat extract medium as originally described by Waksman (1), except that the meat extract was increased from 0.3 to 0.5 per cent. The results are reported in Table I. The purity of the streptomycin sulfate was 400 units per mg., which is somewhat less than that obtained from the Curbay-salts medium but well within the requirements for clinical material.

Preparation of Streptomycin Hydrochloride—The hydrochloride may be prepared by the same process as the sulfate if HCl is substituted for H_2SO_4 at the acidification steps. However, streptomycin hydrochloride is more soluble in acetone and precipitation with 75 per cent acetone was found to be incomplete. Better yields were obtained by concentrating the acetone

eluates and precipitating with 80 per cent acetone. This procedure was demonstrated on an aliquot of Lot 141 reported in Table I. A 1.67 per cent aliquot of the carbon cake representing 4.35 liters (750,000 units) of fermentation harvest was suspended in 500 ml. of 60 per cent acetone. Hydrochloric acid was added to maintain pH 2 while stirring was continued for 60 minutes. The carbon was collected on a Büchner filter and washed with acid acetone. The eluate and washings (915 ml.) were adjusted to pH 7.7 with NaOH and concentrated *in vacuo* below 30° to 85 ml. 4 volumes of acetone (340 ml.) were added to precipitate the streptomycin hydrochloride. After the mixture was chilled overnight, the precipitate was collected by filtration, redissolved in water, shell-frozen, and dried *in vacuo*. Streptomycin hydrochloride was obtained as a slightly yellow powder weighing 860 mg. and assaying 380 units per mg., giving an over-all yield of 327,000 units or 43.5 per cent.

Streptomycin hydrochloride was also prepared from the sulfate. 1 gm. of streptomycin sulfate (632,000 units) was dissolved in 7 ml. of water at pH 3.2. A solution of CaCl_2 (100 mg. per ml.) was added dropwise with constant stirring to a point of no further precipitation. The precipitate was removed by centrifuging and washed. The supernatant solution plus washings was shell-frozen and dried. The streptomycin hydrochloride was obtained as 905 mg. of white powder assaying 590 units per mg., representing a yield from the sulfate of 84 per cent.

Chromatographic Purification of Streptomycin Hydrochloride over Aluminum Oxide—Streptomycin hydrochloride, prepared directly from the fermentation harvests or from the sulfate, was purified further by a chromatographic procedure. The method is similar to that described by Carter *et al.* (3), except that the aluminum oxide was washed with HCl instead of H_2SO_4 . This procedure eliminates the streptomycin sulfate band in the chromatogram. Usually 80 per cent or more of the starting activity can be recovered from the column. The most active fractions have been shown to approach purity by their assay values and have readily yielded the crystalline reineckate (4) and the trihydrochloride-calcium chloride double salt (6).

Preparation of Column—5.5 kilos of Harshaw alumina (No. 2-350, catalyst) were adjusted to pH 4.7 with hydrochloric acid and thoroughly washed with distilled water. The acid-adjusted aluminum oxide was then suspended in 80 per cent methanol and poured, as a slurry, into a glass pipe, 4 inches in diameter producing, after settling, a column 25 inches high.

Procedure—64 million units of streptomycin hydrochloride (400 units per mg.) were dissolved in water and brought to pH 5.8 (final volume 350 ml.). The aqueous solution was diluted with 4 volumes of methanol before passage over the column. Development was carried out with 80 per cent methanol.

In order to obtain solid streptomycin fractions, the alcohol was removed *in vacuo* and the residues were dried from the frozen state. The results are given in Table II.

The potencies of Fractions 11 and 12 (Table II) reduced by 3 per cent to convert them to micrograms per mg. give values of 965 and 854, respectively. These are 14.9 per cent and 1.7 per cent above the theoretical potency of 840 γ per mg. for the pure trihydrochloride. In several instances fractions have been obtained which assay above the theoretical value for the pure trihydrochloride as measured in terms of the current standard. This may be an assay error but it is believed that further work is necessary to explain the discrepancy.

Preparation of Streptomycin Reineckate and Calcium Chloride Double Salt—A reineckate was prepared by the method described by Fried and Wintersteiner (4), starting with a streptomycin hydrochloride of approxi-

TABLE II

Chromatographic Separation of 64 Million Units of Streptomycin Hydrochloride (400 Units per Mg.) over Alumina Column

Fraction No. (300 ml. each)	Units	Solids	Purity	Recovery
	millions	gm.	units per mg.	per cent
10	4.3	5.12	840	6.7
11	20.0	20.17	995	31.2
12	17.4	19.85	879	27.2
13	9.6	16.00	600	15.0
14	6.7	14.10	475	10.5

mately 900 units per mg. After two recrystallizations the crystals melted at 160–163° (corrected) and assayed 400 units per mg.

Crystalline streptomycin trihydrochloride-calcium chloride double salt (6) was prepared as follows: 1 gm. of streptomycin hydrochloride, assaying approximately 900 units per mg., was dissolved in 5 ml. of methanol. To this solution were added 4 ml. of methanol containing 0.466 gm. of calcium chloride. 1 drop of concentrated HCl was added and the mixture cooled in a refrigerator. After 48 hours, 0.443 gm. of crystals was collected, which assayed 828 units per mg.

Application of Carbon-Acetone Method to Streptothricin—*Streptomyces lavendulae* (Waksman Strain 14) was grown in submerged culture on Waksman's dextrose-tryptone medium with added calcium carbonate and nitrate. Assays were run by the same method (8) with a standard streptothricin powder which had been defined in Waksman units. Fermentation harvests assaying 100 to 170 units per ml. were extracted by the process

described above for streptomycin sulfate. The dried streptothricin sulfate was obtained at a potency of 180 to 214 units per mg. with an over-all yield of 40 to 57 per cent.

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SUMMARY

A method is described for the preparation of streptomycin sulfate and streptomycin hydrochloride. The method lends itself to pilot scale operation and the salts are of satisfactory purity for clinical investigations. The essential steps are decolorization with carbon at pH 2, adsorption on carbon at pH 7, elution with dilute acetone at pH 2.5, and precipitation by increasing the acetone concentration to 75 or 80 per cent. The method is applicable to streptothricin. Further purification of streptomycin hydrochloride by means of an aluminum oxide chromatogram is described.

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THE METABOLISM OF L(+)-ARGININE IN THE RAT*

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In a series of papers from this laboratory the extensive metabolic activity of amino acid nitrogen, studied with the aid of N^{15} , has been described. The data have been interpreted to show that following absorption an amino acid interacts with the tissues by replacing the identical acid in the proteins and, through the processes of deamination, reamination, and transamination, yields its nitrogen to build up other protein constituents for protein synthesis. After the administration of isotopic α -amino nitrogen in the form of tyrosine or leucine, a large part of the isotope contained in the tissue proteins was located in amino acids different from those fed (1), indicating that extensive nitrogen transfer from one carbon chain to another had occurred.

Since in the intermediary metabolism of the rat the amidine group of arginine is ultimately derived from α -amino nitrogen, it seemed of interest to follow the metabolic fate of the amidine group of arginine and to compare its reactivity and pathways with those of the α -amino group in amino acids. With the aid of the isotope technique the rapid intermediary formation of the amidine group of arginine has been revealed (2); the findings give support to the theory of the ornithine cycle proposed by Krebs and Henseleit (3). Ornithine arising from arginine by arginase action enters into combination with CO_2 and ammonia or amino acid nitrogen to regenerate the amidine group. The only reactions in which the amidine group of arginine is known to participate are the formation of urea and the transfer of the amidine group to glycine in creatine formation (4, 5).

By feeding rats a preparation of arginine containing heavy nitrogen in the amidine group it was possible to trace this part of the arginine molecule in the mammalian organism. In the present investigation we have determined the quantitative distribution in tissues and excreta of heavy nitrogen after its administration in the form of arginine, labeled with N^{15} in the amidine group. Data on the total arginine content of a rat were obtained by employing the isotope dilution technique. The lack of utilization of

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urea has been confirmed by feeding isotopic urea to rats, when only insignificant amounts of heavy nitrogen were found to be incorporated into nitrogenous tissue constituents.

EXPERIMENTAL

Feeding of l-Arginine Monohydrochloride—Two adult rats, having a combined weight of 646 gm., were kept in separate metabolism cages on a stock diet, containing 15 per cent casein, 68 per cent corn-starch, 5 per cent yeast, 4 per cent salt mixture (6), 2 per cent cod liver oil, and 6 per cent Wesson oil. Each rat received in addition to 15 gm. of stock diet daily for 3 days 86 mg. of *l*(+)-arginine monohydrochloride (8.25 atom per cent excess N^{15} (7)). Urine and feces were collected during the experimental period and the animals were killed by exsanguination. The pro-

TABLE I

Balance of Nitrogen Isotope after Feeding Isotopic l(+)-Arginine Monohydrochloride

The values were calculated from the total nitrogen of the fractions and their isotopic concentrations. The animals consumed 809 microequivalents of N^{15} .

	Microequivalents N^{15}	Fraction of N^{15} administered
		<i>per cent</i>
Excreta, feces.....	9	1.1
“ urine.....	423	52.2
Animal body, non-protein N.....	66	8.2
“ “ protein N.....	234	28.9
Total isotope recovered.....	732	90.7

cedures used in the isolation of nitrogenous compounds from tissues and excreta (Table I) were those employed routinely in this laboratory.

Excreta—The 3 day urine samples of one rat were pooled; they contained 0.781 gm. of nitrogen with 0.379 per cent N^{15} excess; *i.e.*, 211.5 microequivalents of N^{15} . Since one rat received a total of 405 microequivalents of N^{15} , this corresponds to 52.2 per cent of the administered isotope.

24 hour samples were collected from the urine of the second rat and analyzed for urea and ammonia. The N^{15} concentrations were as follows (atom per cent excess):

	1st day	2nd day	3rd day
Urea.....	0.312	0.393	0.439
Ammonia.....	0.038	0.045	0.054

Allantoin—Allantoin was isolated from the pooled urines according to Wiechowsky (8). It contained 0.010 atom per cent excess N^{15} . The combined feces excreted during the entire experimental period contained

9.9 microequivalents of N^{15} , amounting to 1.1 per cent of the administered isotope.

Investigation of Organs—Total protein nitrogen and non-protein nitrogen and their isotope contents were determined in a number of organs and in the remaining carcass. The analytical data as well as those of some amino acids isolated from the protein hydrolysates are listed in Tables II and III.

The isolation of creatine from muscle and its degradation into ammonia and sarcosine were described previously (9).

Hemin—To the blood obtained by heart puncture, oxalate was added and the red blood cells separated by centrifugation. Hemin was isolated according to Moerner (10). The crystals contained no significant excess of N^{15} (0.003 per cent).

Feeding of Urea—Two adult male rats having a combined weight of 562 gm. received the regular stock diet and each rat received a daily addition of

TABLE II
Atom Per Cent Excess N^{15} in Protein and Arginine Nitrogen of Tissues from Rats
Given Isotopic Arginine

Organ	Protein N	Arginine N
Serum.	0.023	0.153
Liver ..	0.027	0.113
Intestinal tract	0.111	0.597
Kidney	0.059	0.347
Combined hearts, lungs, testes, spleens	0.042	0.250
Carcass .	0.016	0.099

50 mg. of isotopic urea (15.2 atom per cent excess N^{15} (7)) for 3 days. Urea and ammonia were isolated from the pooled urines, and some of the body constituents analyzed. The analytical data are given in Table IV.

Determination of Arginine in Proteins of Rat—The isotope dilution method (11) was employed for determining the total amount of arginine in rat proteins. To an aliquot of a hydrolysate from an entire rat carcass containing 0.415 gm. (29.64 milliequivalents) of nitrogen were added 26.32 mg. (0.125 mm) of *l*(+)-arginine monohydrochloride containing 8.25 atom per cent N^{15} excess. Arginine was precipitated as the flavianate. After two recrystallizations, flavianic acid was removed in the usual manner and the amino acid converted into the *p*-toluenesulfonyl derivative; m.p. 256°.

Calculated, N 17.1; found, N (Kjeldahl) 17.0

It contained 0.868 atom per cent N^{15} excess. The amount of arginine present in the body may therefore be calculated from the expression $((8.25/$

0.868)—1) $0.125 = 1.063$ mm of arginine = 4.252 milliequivalents of arginine nitrogen. Of the total nitrogen 14.34 per cent was therefore arginine nitrogen. This corresponds to an arginine content of $(14.34/6.25) \times (174/56) = 7.13$ per cent in the total carcass protein.

Calculation of Deposition of Arginine—In the present experiment the total nitrogen content of the tissues analyzed was 17.92 gm. According to the above arginine determination, 14.34 per cent = 2.57 gm. of this were arginine nitrogen. The N^{15} content of the arginine isolated from the carcasses of the rats fed isotopic arginine was 0.099 per cent N^{15} . Since 93 per cent of the total protein of the animal was present in the carcass, this isotope concentration can be assumed to be close to the average N^{15} value of the arginine of the entire animal. The organ proteins contained a total of 234 microequivalents of N^{15} . Assuming that of the total protein nitrogen (17.92 gm.) 14.34 per cent = 2.57 gm. was arginine nitrogen, then $(2.57 \times 0.099) / (100 \times 14) = 182$ microequivalents of N^{15} were present as arginine nitrogen; i.e., 78 per cent of the total N^{15} in the protein nitrogen is due to its content of isotopic arginine. This value is increased to 86 per cent if the isotope concentration of arginine in the individual organ proteins is taken into account and if it is assumed that the arginine content of the individual proteins is the same as the average value of 7.13 per cent.

DISCUSSION

Deposition of Arginine—The rats had received an addition of isotopic arginine to the stock diet for 3 days. As shown in Table I, 52.2 per cent of the ingested isotope was excreted during the experimental period, while only 29 per cent was incorporated in the various organ proteins. In the present experiment, the uptake of heavy nitrogen resulted almost entirely from the replacement of arginine in the tissue protein by dietary arginine. 86 per cent of the total quantity of isotope in the animal protein could be accounted for by the isotope content of arginine. This figure was reached by determining the total protein nitrogen, total isotope content of the protein, arginine content in the carcass protein, and isotope concentration of the arginine samples isolated from the proteins. The finding that practically the entire heavy nitrogen in the protein was present as isotopic arginine showed clearly that very little, if any, of the amidine nitrogen had been used for amino acid synthesis. In contrast, when isotopic leucine or tyrosine had been fed, less than one-third of the total isotope in the proteins resulted from deposition of the ingested amino acid proper, while the remaining isotope in the protein was due to nitrogen transfer to other carbon chains (1, 2). It thus appears that when α -amino nitrogen and ammonia have entered into the ornithine cycle, they are no longer available

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for intermediary protein metabolism. In this respect the amidine shift of arginine to form creatine is no exception, as, apart from the reaction involving phosphoric acid, the metabolism of creatine in mammals appears to be restricted to the formation and immediate elimination of creatinine (12). The fact that the amidine group contributes but little to the general metabolic pool of nitrogen is further illustrated in the present experiments

TABLE III
Atom Per Cent Excess N¹⁵ in Amino Acids and Other Nitrogenous Constituents from Rats Given Isotopic Arginine

	Liver	Combined internal organs (heart, lungs, spleen, testes)
Arginine.....	0.113	0.250
Amide.....	0.014	
Glutamic acid.....	0.030	0.010
Aspartic ".....	0.017	0.006
Histidine.....		0.010
Lysine.....		0.108
Allantoin from urine.....		0.003
Creatine (muscle).....		
Hemin.....		

TABLE IV
Atom Per Cent Excess N¹⁵ in Excreta and Tissue Constituents of Rats Given Isotopic Urea

Urinary urea.....	1.250
" ammonia.....	0.051
Liver total protein N.....	0.020
" arginine.....	0.027
Arginine in protein of intestinal tract.....	0.020
Kidney arginine.....	0.020
Muscle creatine.....	0.004

by the lowness of the isotope concentrations found in other amino acids such as glutamic and aspartic acids (Table III).

The presence of isotope in urinary ammonia, amide nitrogen, and amino acids such as glutamic and aspartic acids may be due to a conversion of urea to ammonia, possibly by bacterial enzyme. This reaction is quantitatively insignificant and must be very slow. A similar decomposition to ammonia was observed when isotopic urea was fed to rats (Table IV). No isotope enters the creatine molecule when administered in the form of urea (Table IV).

The utilization of amidine nitrogen for purine synthesis, which appeared possible in view of the presence of N-C-N groups in purines, was not indicated by the experimental data. Allantoin isolated from the urine of the animals which had received isotopic arginine did not contain a significant concentration of N¹⁵.

Reactivity of Various Proteins—In Table II are given the isotope concentrations of arginine samples isolated from a number of organ proteins. As the isotope content of the proteins is preponderantly due to the presence of marked arginine, the N¹⁵ concentration in this amino acid forms a measure of reactivity of the various proteins. It will be noted that the protein of the intestinal wall had the highest isotope concentration. High concentrations were also present in kidney protein and in a fraction containing the combined proteins of heart, lung, spleen, and testes. The values are appreciably lower in the proteins of liver, serum, and muscle. On comparison of these data with the chemical activity of organ proteins, as measured by isotope uptake, after administration of leucine, some marked differences are evident. In the present experiment the activity of liver and serum proteins did not differ substantially from that of the muscle proteins, whereas after leucine feeding the proteins of liver and serum were regenerated 2 to 3 times faster than muscle protein (2).

The low level of heavy nitrogen concentration in liver protein after the ingestion of arginine with a marked amidine group may reasonably be ascribed to the action of arginase, which in mammals appears to be localized in the liver. The isotopic dietary arginine on entering the liver cells will be acted upon by arginase, leading to a replacement of most of the isotopic amidine nitrogen by ordinary nitrogen from other sources. The low reactivity of the liver protein, judged by its isotope content, is apparent only and reflects merely the rapid rate at which the ornithine cycle is operating in the liver. Likewise, the small isotope concentration in the serum proteins contrasts with the findings obtained after the feeding of isotopic α -amino nitrogen in the form of leucine, when the serum proteins contained the highest isotope concentration of all organs analyzed. In order to explain the low value, it may be assumed either that serum proteins are produced in the liver or that they are manufactured from the same metabolic pool of nitrogen which is utilized for the synthesis of liver proteins; i.e., the arginine employed had previously entered the ornithine cycle. This finding is in agreement with the view that serum proteins are made in the liver.

If the insignificant, lymphatic route is disregarded, the bulk of the absorbed amino acids would be carried by the portal vein and enter the various internal organs only after passage through the liver. The present

finding, that the arginine in proteins of kidney and other internal organs contained an isotope concentration several times that of the liver arginine, illustrates the rapid passage of amino acids through the liver.

The action of arginase involves primarily that fraction of the circulating arginine which enters the liver cells and is utilized for synthesis of liver proteins, while the arginine transported to other internal organs appears to remain relatively intact. If the fate of dietary arginine is taken as an indicator of a general reaction, it would seem that of the amino acid mixture carried by the portal vein, only that fraction is retained by the liver which is required for the regeneration of liver and possibly serum proteins.

An alternative explanation may be considered for the high isotope uptake observed in the protein of the intestinal wall. The rapidly regenerating protein, particularly that of the intestinal mucosa, may utilize the amino acids while they are being absorbed. The dietary isotope would then not be diluted appreciably by ordinary nitrogen arising from intermediary metabolism.

Metabolism of Urea—The inability of the animal organism to employ urea for intermediary reactions had already been indicated by the behavior of the urea precursor, the amidine group of arginine. Thus, the results obtained from the feeding of isotopic urea to rats likewise illustrate the lack of any metabolic activity of this compound. A participation of urea in any intermediary reactions is not indicated. Specifically, and in contrast to the amidine group of arginine, urea is not a precursor of creatine, as had been claimed (13). Utilization of urea in purine formation may also be ruled out. When potential urea was supplied to animals in the form of arginine containing an isotopic amidine group, the allantoin isolated from the urine did not contain a significant excess of heavy nitrogen.

Small isotope concentrations appear in body proteins and urinary ammonia after urea feeding (Table IV). In view of the high isotope content of the administered material they become, however, rather insignificant and cannot be the result of a utilization of urea. As in the arginine feeding experiment, these values indicate a slow decomposition of urea in the animal body, possibly due to bacterial action in the gut.

SUMMARY

1. *l*(+)-Arginine containing heavy nitrogen in the amidine group was administered to rats and the distribution of the isotopic label was studied in excreta and body constituents.

2. The data obtained indicate a rapid regeneration of the amidine group of arginine in the liver, in agreement with the theory of the ornithine cycle.

3. No evidence was obtained that the amidine nitrogen of arginine can be utilized for any reactions of intermediary metabolism except for creatine formation.

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THE METABOLISM OF L(+)-ARGININE AND SYNTHESIS OF CREATINE IN THE PIGEON*

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In mammals, arginine occupies a unique position among amino acids as a vehicle for nitrogen in the catabolism of proteins. Nitrogen arising from amino acid breakdown becomes incorporated into the amidine group of arginine, which is subsequently hydrolyzed to ornithine and urea. The enzyme arginase is an essential component of the cyclic system responsible for urea formation. It is improbable that the arginine-ornithine cycle is of similar significance for birds because in this animal class (a) uric acid is the principal product of nitrogen catabolism, (b) arginase is not present in the avian liver, and (c) arginine is an indispensable amino acid for the bird (1). Since ornithine cannot replace arginine in avian nutrition (2), it would appear that birds are unable to convert ornithine to arginine. The reverse reaction, conversion of arginine to ornithine, must occur, since in birds benzoic acid is coupled with ornithine to form ornithuric acid. The process yielding ornithine does not necessarily involve the action of arginase. Ornithine could conceivably result from transference of the amidine group of arginine to some other compound, a reaction which is known to occur in the biological synthesis of guanidoacetic acid (3, 4).

In view of the differences of arginine metabolism in mammals and birds it seemed of interest to compare the fate of this amino acid in the two animal classes. When arginine containing an amidine group labeled with N^{15} was administered to rats, the arginine isolated from liver protein had an isotope level which was small in comparison to that of arginine in the proteins of other internal organs (5). The finding was taken to illustrate the localized action of arginase on liver arginine which resulted in a rapid replacement of isotopic nitrogen in the amidine group by normal nitrogen during the re-synthesis of arginine. On the other hand, arginine from the liver of pigeons which had received corresponding amounts of similarly labeled arginine contained a greater excess of isotopic nitrogen than the arginine of any other organ examined (Table I). Hence, regeneration of arginine could

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not have occurred at a significant rate, a finding in accord with the essential nature of arginine in this animal species. Small quantities of arginase can be found in avian kidney (6). Since the arginine from kidney protein contained nearly the same concentration of N^{15} as that from liver protein, arginase action cannot have been extensive in the kidney. In view of the limited occurrence of arginase in the avian organism, it may be asked in what manner arginine is metabolized in birds. The excreta of pigeons contain small quantities of urea which have been reported to increase following the administration of arginine (7). In order to investigate whether urea formation can account for the catabolism of the amidine portion of arginine, the distribution of N^{15} was studied in the excreta of pigeons which had re-

TABLE I

Atom Per Cent Excess N^{15} in Tissue Constituents from Pigeons Given Isotopic l(+)-Arginine Monohydrochloride

	Liver	Kidney	Intestinal tract	Carcass
Total protein N.....	0.070	0.029		0.023
Purine N.....	0.040	0.029		
Amide ".....	0.053	0.070	0.043	0.024
Arginine.....	0.427	0.376	0.222	0.121
Glutamic acid.....	0.021			

TABLE II

Atom Per Cent Excess N^{15} in Excreta from Pigeons Given Isotopic Arginine

	Experiment I	Experiment II	
			Fraction of total N^{15} excreted <i>per cent</i>
Total N of excreta		0.082	100.0
Urea.....	2.63	0.848	51.7
Uric acid	0.092	0.022	16.0
Unidentified portion of alcohol extract		0.286	32.6

ceived labeled arginine. Of the total isotopic nitrogen administered only 27 per cent was excreted during the 3 day experimental period. This compares with a value of 52 per cent found in the urine of rats which had been given the same quantity of labeled arginine. The greater retention of arginine by the bird is a reflection of its inability to obtain this amino acid from endogenous sources. The urea, which comprised 5 per cent of the total excretory nitrogen, had an isotope concentration accounting for 52 per cent of the total N^{15} excreted (Table II). The level of isotope in

urea is high compared to that of liver or kidney arginine, suggesting that it may have been partly of bacterial rather than of metabolic origin. The isotope concentration in uric acid was considerably lower and accounted for 15 to 20 per cent of the total N^{15} eliminated. Although small compared to urea, the isotope level in uric acid exceeds that of any other compound isolated with the exception of arginine. Urea is probably not responsible for the appearance of N^{15} in uric acid, since Barnes and Schoenheimer found only an insignificant utilization of urea for purine and uric acid synthesis in pigeons (8). It is likely that small amounts of isotopic ammonia were formed from the amidine group of arginine. In both liver and kidney protein the isotope level in the amide nitrogen, which may be taken as a measure of ammonia production, was only somewhat lower than in the uric acid. Ammonia, which is readily utilized by the pigeon for uric acid formation (8), may have been responsible for the incorporation of N^{15} into uric acid. Nevertheless it should be pointed out that in the analogous rat experiment, allantoin, the end-product of purine metabolism, contained no significant excess of N^{15} (5). A direct utilization of the amidine group of arginine in purine and uric acid synthesis is therefore not excluded. The amidine group of arginine has frequently been suggested as the direct precursor of purines and uric acid. The results obtained in the present experiment are not inconsistent with this view, but they suggest that if such a pathway exists it can account only for a fraction of the arginine catabolized. Since not more than half of the total isotopic nitrogen was excreted in the form of urea, it is likely that end-products other than urea are formed from the amidine group. Some evidence has been obtained in the present experiment that the excreta contain a nitrogenous fraction which has a relatively high isotope level, but consists neither of urea nor uric acid (Table II). That this fraction is not creatine may be inferred from the much smaller isotope concentration of muscle creatine.

It has been reported (9) that in the chick citrulline but not ornithine can fulfil the arginine requirements for growth. If the bird were able to aminate citrulline to form arginine, then the administration of isotopic ammonia should lead to an incorporation of N^{15} in the amidine group of arginine. This was not the case in the investigation carried out by Barnes and Schoenheimer with pigeons (8). The negative finding has been confirmed in the present investigation. It is conceivable that this failure is due to the fact that citrulline is not normally formed in birds but can be converted to arginine if supplied with the diet. The excess N^{15} found in liver or kidney arginine was not significantly greater when both citrulline and heavy ammonium citrate were administered than it was without citrulline addition. Evidently, the pigeon is unable, even in the presence of citrulline, to synthesize the amidine group of arginine, unless ammonia is not the direct or indirect source of nitrogen for this amination. It would be of interest to

ascertain whether the contrasting results obtained in growth experiments with chicks and in the present experiments with pigeons signify differences of the two animal species in the utilization of citrulline.

Metabolism of Creatine in Pigeon—The mechanism of biological creatine formation has been elucidated mainly with the rat as the experimental animal. Whereas the methylation of guanidoacetic acid to creatine has been clearly demonstrated in the pigeon (10), little direct evidence exists relative to the process of guanidoacetic acid formation in this animal species. The muscle creatine isolated from pigeons which had received amidine-labeled arginine contained only about one-third of the isotope concentration found in creatine from the corresponding rat experiment (Table III). This finding was unexpected, because in the absence of arginase the

TABLE III
Atom Per Cent Excess N¹⁵ in Muscle Creatine from Rats and Pigeons

Isotopic amino acid administered	Pigeon			Rat (cf. (3))		
	Total creatine N	Amidine N	Sarcosine N	Total creatine N	Amidine N	Sarcosine N
Arginine	0.030	0.047	0.002	0.108	0.130	0.003
	0.65	1.00	0.04	1.90	2.21	0.05
Glycine	0.052	0.016	0.128	0.030	0.006	0.070
	0.54	0.17	1.35	1.00	0.02	2.26
Ammonia	0.035	0.013	0.077	0.036	0.044	0.014
	0.39	0.15	0.86	1.64	2.0	0.64

The quantities of amino acid and their N¹⁵ concentrations differed in the various feeding experiments. For comparison the N¹⁵ concentrations obtained in creatine and its degradation products are recalculated on the assumption that the total quantity of N¹⁵ administered was the same in all experiments. The bold-faced figures are the values of (atom % excess N¹⁵ formed \times 100)/(mg. N¹⁵ administered).

amidine group of the labeled arginine should have retained most of its isotopic nitrogen. Under identical conditions, the isotope level of the creatine precursor should have been higher in the pigeon than in the rat. The comparatively poor efficiency of arginine as a creatine precursor in the bird could be the result of either a slower rate of synthesis or it could be evidence for the existence of more than one source of nitrogen for the amidine group of creatine.

For the pigeon, as for the rat, the main source of nitrogen for the sarcosine moiety of creatine seems to be glycine. Judged from the incorporation of heavy nitrogen after administration of labeled glycine, this amino acid is about two-thirds as efficient a precursor as in the rat (Table III). The quantitative differences in arginine and glycine utilization for creatine

synthesis in the mammalian and avian organism may therefore be attributed, at least in part, to kinetic factors. The existence of additional precursors is, however, not excluded. It may be mentioned that Borsook and Dubnoff observed a small but significant effect of glycine on the synthesis of guanidoacetic acid in slices of pigeon kidney, but found arginine to be inefficient (11). All organs of the pigeon examined showed a much slower rate of guanidoacetic acid synthesis than those of the rat.

In rats, ammonia nitrogen is utilized for the formation of the amidine moiety of creatine through the amidine group of arginine as an intermediate. Since this grouping cannot be synthesized by the pigeon, heavy nitrogen administered in the form of ammonia should fail to appear in the amidine moiety of creatine. Barnes and Schoenheimer, using ammonia which had a relatively small isotope concentration, found only an insignificant excess of isotope in creatine (8). In the present experiment, the creatine isolated after administration of labeled ammonia had an appreciably higher isotope level, but this was largely in the sarcosine part, and not in the amidine moiety (Table III). This finding eliminates as a primary source of the amidine group of creatine any nitrogenous compound which can be directly formed from ammonia.

Isotope analysis of the sarcosine derived from creatine reveals that ammonia nitrogen had been utilized for its synthesis. If, as in the rat, glycine was the main source of this grouping, it would follow that ammonia furnished nitrogen for glycine synthesis. Since in the growing chick glycine is required for optimal growth, this animal species appears to have a limited ability to synthesize glycine (12). From the data presented here it appears that in the pigeon synthesis of glycine takes place and that lack of nitrogenous precursors cannot be responsible for any inadequacy of such synthesis.

EXPERIMENTAL

The preparation of *l*(+)-arginine monohydrochloride containing N^{15} in the amidine group has been described previously (13). A sample of isotopic glycine was generously supplied by Dr. D. Shemin of this Department.

Feeding Experiments

Arginine Feeding, Experiment I—Two pigeons having a combined weight of 618 gm. were kept on an adequate diet of mixed grains. *l*(+)-Arginine monohydrochloride containing 16.5 atom per cent excess N^{15} in the amidine group was dissolved in water and administered by stomach tube in 6 hour intervals for 3 days. The two animals received a total of 414 mg. of arginine monohydrochloride, corresponding to 9.1 mg. of N^{15} or 650 microequivalents of N^{15} . At the end of the experimental period the pigeons were

killed by decapitation. Samples of purine nitrogen were obtained from liver and kidney protein by the method of Graff and Maculla (14). Arginine and glutamic acid were obtained by procedures routinely employed in this laboratory. Isolation of muscle creatine in the form of creatinine picrate and degradation of creatinine to ammonia and sarcosine were carried out as described previously (13). The isotope concentrations of various nitrogenous tissue constituents are listed in Tables I and III.

From the pooled droppings excreted during the experimental period, uric acid was isolated by the method of Neubauer and Huppert (15). For the isolation of urea a portion of dry finely ground droppings was extracted continuously for 8 hours with 90 per cent ethanol containing 1 per cent acetic acid. The extract was brought to dryness *in vacuo* and the residue dissolved in water. Urea was precipitated from this solution as the dioxanthryl compound and the latter recrystallized from glacial acetic acid. The N^{15} concentrations of uric acid and urea are given in Table II.

Arginine Feeding, Experiment II; Distribution of N^{15} in Excreta—One pigeon weighing 320 gm. received by stomach tube over a period of 2 days a total of 217 mg. of *l*(+)-arginine monohydrochloride in aqueous solution. The compound contained 4.20 atom per cent excess N^{15} in the amidine group, corresponding to a total of 86.5 microequivalents of N^{15} . The (air-dried) droppings excreted during the experimental period weighed 5.58 gm. and contained 7.16 per cent nitrogen. The N^{15} concentration was 0.082 atom per cent excess N^{15} . Therefore a total of $(5.58 \times 7.16 \times 0.082 \times 100)/14 = 23.4$ microequivalents or 27 per cent of the total N^{15} administered was excreted during the experimental period. In order to determine the quantitative distribution of N^{15} among the nitrogenous constituents of the droppings it was necessary to obtain analytical data on the composition of pigeon excreta. Since the values reported for the uric acid content of various species of birds differ considerably (16), a quantitative determination was carried out by the isotope dilution method. 3 gm. of droppings from a normal pigeon on a non-isotopic diet were finely ground and intimately mixed with 0.300 gm. of uric acid containing 0.092 atom per cent excess N^{15} . The mixture was extracted exhaustively by hot 2 per cent NaOH and uric acid isolated from the extract by the method of Neubauer and Huppert (15). After several reprecipitations from alkaline solution with hydrochloric acid the uric acid (N(Kjeldahl) found 33.3, calculated 33.3 per cent) contained 0.042 atom per cent excess N^{15} . The 3 gm. of droppings which contained 201 mg. of N therefore contained $0.300 \times (0.092/0.042 - 1) = 0.357$ gm. of uric acid, corresponding to 119 mg. of uric acid nitrogen or 59.2 per cent of the total nitrogen.

If this value is used, then 59.2 per cent of 0.400 gm. = 0.237 gm. was uric acid nitrogen in the present experiment. The uric acid excreted after ad-

ministration of isotopic arginine contained 0.022 atom per cent excess N^{15} . Therefore, $(0.022 \times 0.237)/14 = 3.7$ microequivalents of N^{15} , or 16 per cent of the total N^{15} excreted, were present as uric acid.

For a quantitative determination of urea a portion of the droppings was extracted exhaustively with 90 per cent ethyl alcohol containing 1 per cent acetic acid. The extract was evaporated to dryness *in vacuo* and taken up in H_2O . Urea was precipitated from this solution as dioxanthryl urea. Three separate determinations gave 4.82, 4.82, and 5.6 per cent of urea nitrogen in the total nitrogen of the droppings. The urea nitrogen contained 0.848 atom per cent excess N^{15} . Of the total nitrogen excreted (0.400 gm.) $(5.0 \times 0.400)/100 = 20$ mg. were urea nitrogen; hence $(20 \times 0.848)/14 = 12.1$ microequivalents of N^{15} , or 51.7 per cent of the total N^{15} excreted, were present as urea. The isotope content of urea and uric acid accounts for 68 per cent of the total N^{15} excreted. The remaining 32 per cent could not be identified. The alcohol extract of the dried pigeon droppings contained 58 mg. of nitrogen with an isotope concentration of 0.480 atom per cent excess N^{15} . This corresponds to 14.5 per cent of the total nitrogen excreted and 83.5 per cent of the N^{15} present in the droppings. Urea nitrogen (20.0 mg.) accounted for 34.5 per cent of the nitrogen and for 61 per cent of the N^{15} in the alcohol extract. The remaining nitrogen (38.0 mg.) must have contained an average concentration of $((0.48 \times 58) - (20 \times 0.848))/38.0 = 0.286$ atom per cent excess N^{15} . The values for the isotope concentrations in the nitrogenous portions of the excreta are given in Table II.

Feeding of Glycine—One pigeon, weighing 285 gm., received by stomach tube three doses daily of isotopic glycine for a period of 3 days. A total of 150 mg. of glycine containing 33.8 atom per cent excess N^{15} was given, corresponding to 9.45 mg. or 675 microequivalents of N^{15} . At the end of the experimental period the pigeon was killed and the creatine isolated from muscle. The N^{15} concentrations are given in Table III.

Feeding of Ammonium Citrate and Citrulline—One pigeon weighing 300 gm. received by stomach tube a total of 61.7 mg. of NH_3 as ammonium citrate containing 14.5 atom per cent excess N^{15} and 180 mg. of *dl*-citrulline (17). The solution was administered in three doses daily over a period of 3 days. At the end of the experimental period the pigeon was killed and arginine isolated from liver and kidney protein. The isotope concentrations were for liver arginine, total N 0.016, amidine N 0.020; for kidney arginine, total N 0.019, amidine N 0.025 atom per cent excess N^{15} . The isotope data for muscle creatine are given in Table III.

Feeding of Ammonium Citrate Alone—One pigeon received the same quantity of isotopic ammonium citrate as in the first experiment, but no citrulline. The arginine isolated contained for liver arginine, total N

0.009, amidine N 0.008; for kidney arginine, total N 0.009, amidine N 0.009 atom per cent excess N¹⁵.

SUMMARY

1. *l*(+)-Arginine containing nitrogen N¹⁵ in the amidine portion was administered to pigeons and the distribution of isotope was determined in the nitrogenous fractions of the excreta and in various tissue constituents.

2. In the pigeon, the amidine group of arginine cannot be synthesized, since this moiety contained no excess of N¹⁵ when isotopic ammonia alone or ammonia and citrulline were administered.

3. The distribution of isotopic nitrogen in muscle creatine was determined after administration of labeled arginine, glycine, and ammonia.

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FACTORS INFLUENCING THE INACTIVATION OF UREASE BY ALKYLATING AGENTS*

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In the production of biological effects such as vesication by bis- β -chloroethyl sulfide (mustard gas) it has been considered probable that inactivation of tissue enzymes is of foremost importance. In view of the reaction characteristics of bis- β -chloroethyl sulfide it has appeared likely that this inhibition is due to an alkylation reaction involving essential groups of the enzymes. Involvement of sulfhydryl groups especially has been suggested by knowledge of the high reactivity of bis- β -chloroethyl sulfide with such groups in simple compounds.

To furnish information on some of the factors affecting this mechanism of enzyme inhibition, an investigation and comparison have been made of the influence of hydrogen ion, phosphate, and alkylating agent concentrations, and the state of activity on the inactivation of a single enzyme, urease, by bis- β -chloroethyl sulfide and by several other agents of somewhat differing characteristics. This particular enzyme was chosen, not because of any supposed involvement of urease in vesication, but because many of the physical and chemical properties of urease have already been characterized and its ureolytic activity, which is conveniently measurable, has been well demonstrated to depend upon the freedom of specialized sulfhydryl groups (1).

The alkylating agents considered in this study were bis- β -chloroethyl sulfide, its half hydrolysis product, β -chloro- β' -hydroxydiethyl sulfide (also known as semi-H), and an oxidation product, divinyl sulfone. For comparative purposes inactivation by benzoquinone, 2-methyl-1,4-naphthoquinone, and iodine was also investigated. It was to be expected that differences would be found in the behavior of the chloroethyl sulfides and divinyl sulfone, for although they are all vesicant alkylating agents reacting readily with sulfhydryl groups, they have different reactivities with respect to other groups. There is a distinct contrast in the stabilities of these compounds in water; the chloroethyl sulfides hydrolyze readily with the production of innocuous thiodiglycol and hydrochloric acid, while divinyl sulfone

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

is relatively unreactive with water. Thus the proportion of the chloroethyl compounds which react in water solution with hydroxyl ions, and sulfhydryl, amino, and carboxyl groups is dependent on the individual reactivities and concentrations of each group present. On the other hand, in the case of divinyl sulfone, the reactivities and concentrations of the various amino acid groups determine not only the proportion of divinyl sulfone reacting with each, but also the over-all rate of disappearance of this reagent. The rate of disappearance of divinyl sulfone in aqueous reaction mixtures is usually less than that of the chloroethyl compounds.

Methods

Crystalline urease obtained by aqueous acetone extraction of jack bean meal in the manner described by Sumner (2) was dissolved in various aqueous solutions and routinely incubated for 2 to 3 hours at 30° to obtain a condition of approximately constant ureolytic activity before exposure to inhibiting agents. These agents were added in alcohol solution equivalent in volume to 1 per cent of the final reaction mixture, and complete solution was effected by brief shaking. A period of 15 minutes at 30° was then allowed for complete reaction when the chloroethyl compounds were used. With divinyl sulfone longer times were employed, as specified below. At the end of the reaction period, the extent of inactivation was determined by comparison of the ureolytic activity remaining in the reaction mixture with the activity of a control solution. The ureolytic activity of all solutions was determined in the manner established by Sumner (2); i.e., by mixing the solutions with an equal volume of 3 per cent urea in neutral 0.6 M phosphate buffer and allowing the reaction to proceed for exactly 5 minutes at 20°. At the end of this time the enzymatic activity was arrested by addition of hydrochloric acid. The amount of ammonia produced was measured by a diffusion method in which absorption in glycerol-boric acid and titration to an indicator end-point with hydrochloric acid (3)¹ were employed.

EXPERIMENTAL

*Inhibition by Bis-β-chloroethyl Sulfide*²—The influence of the age of the urease solution on the susceptibility of the enzyme to inhibition was investigated first, because the ureolytic activity was observed to increase for an hour or two from the time the crystalline urease was dissolved in buffer

¹ We have employed an overnight distillation period to insure complete transfer of ammonia to the glycerol-boric acid, and instead of vaseline have used Carbowax 1500 to seal the diffusion vessels, considerably facilitating their cleaning.

² The dichloroethyl sulfide was prepared by reaction of thiodiglycol with thionyl chloride and was purified by vacuum distillation followed by crystallization from ether at a low temperature.

solution. Accordingly, sufficient dichloroethyl sulfide to produce an initial concentration of 0.002 M was added to freshly prepared and 3 hour incubated solutions of urease in 0.025 M phosphate buffer at pH 8.5, 30°. It was found that when the activity was 1.12 Sumner units per ml. in the fresh solution 53.5 per cent inhibition occurred, while in the incubated solution at 1.35 units per ml. activity there was 57 per cent inhibition, a difference which was not considered significant.

The influence of the hydrogen ion concentration on the reaction of dichloroethyl sulfide with urease was investigated by exposure of urease to 0.001 and 0.002 M dichloroethyl sulfide at pH 6, 7, 8, and 8.5 in 0.025 M phosphate buffer. This buffer was adequate to prevent significant change

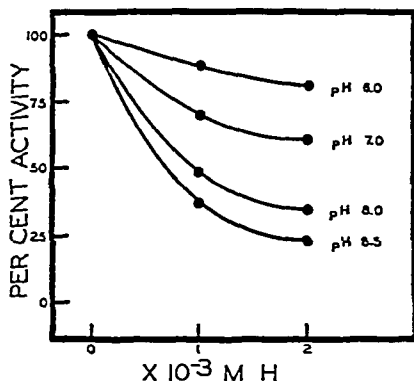


FIG. 1. Curves showing the proportion of ureolytic activity (in per cent) remaining after reaction of urease with different amounts of bis- β -chloroethyl sulfide (H) in 0.025 M phosphate buffer at each of four different hydrogen ion concentrations.

in pH during reaction. Fig. 1 shows that the susceptibility of the enzyme to inactivation increases markedly with increase of the pH from 6 to 8.

The influence of varying the phosphate concentration in the reaction mixture was next investigated. This was done by exposing the enzyme to reaction with 0.0003 M bis- β -chloroethyl sulfide in water and in 0.025 M phosphate, both maintained at pH 8 to 8.5 during reaction by continuous titration with sodium hydroxide under potentiometric observation. Under these conditions there was 91.5 per cent inactivation in the absence of phosphate and only 26.3 per cent in its presence. Further information on the relation of phosphate concentration to inactivation was obtained by exposing urease to 0.001 and 0.002 M dichloroethyl sulfide in 0.025, 0.1, and 0.3 M phosphate buffer solutions all at pH 7. The results are shown in Table I,

from which it is evident that increasing inhibition was obtained with decreasing phosphate concentration.

Inhibition by β -Chloroethyl- β' -hydroxyethyl Sulfide—Urease was treated with several concentrations of β -chloroethyl- β' -hydroxyethyl sulfide³ in the same manner as with the dichloroethyl compound, but only at pH 7 and 8.5, and in a single phosphate buffer concentration of 0.025 M. It will be ob-

TABLE I
Activity of Exposed Urease (in Per Cent)

Initial concentration of bis- β -chloroethyl sulfide M	Phosphate concentration in solution, all at pH 7		
	0.025 M	0.1 M	0.3 M
0	100	100	100
0.001	75	90	96
0.002	55	86	95.5

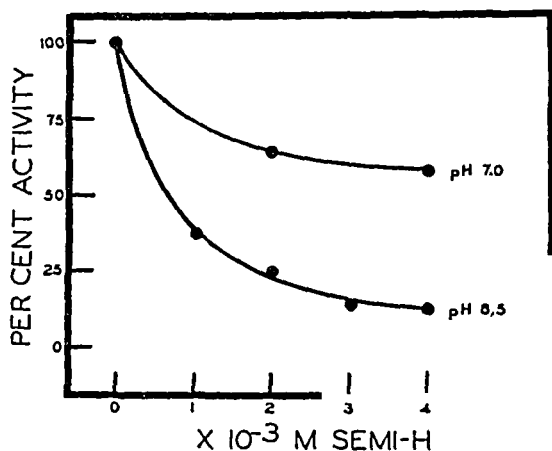


FIG. 2. Curves showing the proportion of ureolytic activity (in per cent) remaining after reaction of urease with different amounts of β -chloroethyl- β' -hydroxyethyl sulfide (semi-H) in 0.025 M phosphate buffer at pH 7.0 and at pH 8.5.

served (Fig. 2) that the inhibitory action of the monochloroethyl compound is approximately half that of the dichloroethyl compound, but that the action of both is similarly influenced by pH.

Inhibition by Divinyl Sulfone—The influence of divinyl sulfone on urease activity was determined by employing the same experimental technique as already described for dichloroethyl sulfide, except that longer times were permitted for reaction, owing to the slower rate of reaction of divinyl

³ A description of the method of synthetic preparation of this compound is in press.

sulfone. Data on the activity of urease following exposure to different concentrations of divinyl sulfone at pH 8 in 0.025 M phosphate buffer for 60 minutes at 30° are presented in Fig. 3. The influence of the pH on the

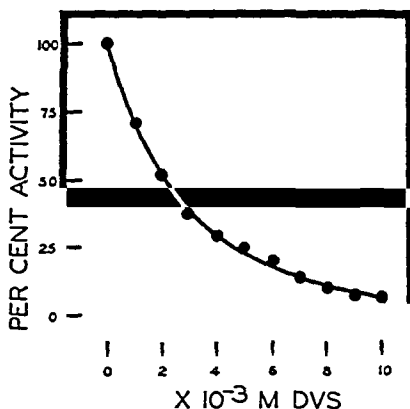


FIG. 3. Curve showing the proportion of ureolytic activity (in per cent) remaining after reaction of urease with different amounts of divinyl sulfone (DVS) for 1 hour in 0.025 M phosphate buffer at pH 8, 30°.

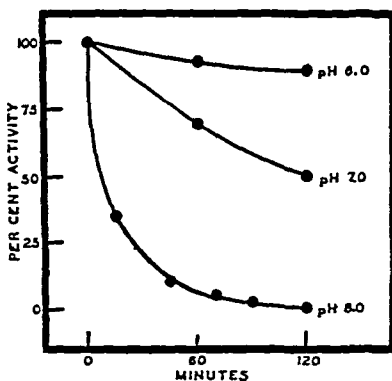


FIG. 4. Curves showing the proportion of ureolytic activity (in per cent) present during the reaction of urease with 0.001 M (initial concentration) divinyl sulfone (DVS) in 0.025 M phosphate buffer at three different hydrogen ion concentrations.

rate of inactivation is shown in Fig. 4 for 0.001 M divinyl sulfone in 0.025 M phosphate buffer at pH 6, 7, and 8 at 30°. The inactivation reaction, like that for dichloroethyl sulfide, is seen to be markedly dependent on the hydrogen ion concentration between pH 6 and 8.

The effect of phosphate on the inhibition of urease by divinyl sulfone was examined by exposing the enzyme to 0.002 M divinyl sulfone for 1 hour at 30° in several different concentrations of phosphate buffer and in sodium

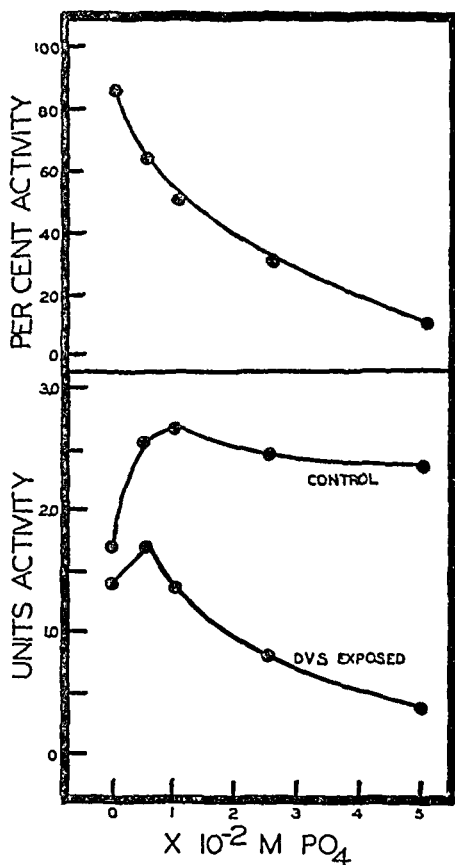


FIG. 5. Lower section, curves showing the ureolytic activity (in units) of solutions of urease in varying concentrations of phosphate buffer at pH 8 to 8.5 at 30° and in similar solutions treated with 0.002 M (initial concentration) divinyl sulfone (DVS) for 1 hour; upper section, curve representing a ratio of the curves of the lower section of the figure and showing the proportion (in per cent) of the ureolytic activity of control urease solutions of varying phosphate concentration after reaction with 0.002 M divinyl sulfone for 1 hour at pH 8 to 8.5, 30°.

hydroxide solution at pH 8 to 8.5. Each of these solutions contained the same concentrations of crystalline urease and, as usual, was incubated first for 3 hours at 30°. The activities measured in control and divinyl sulfone-exposed solutions are shown in Fig. 5. It will be observed that the activity

of the control varied somewhat with the amount of phosphate present. The per cent inactivation caused by divinyl sulfone at each phosphate concentration is also shown as a part of Fig. 5 in which it may be seen that, in direct contrast to the behavior observed with dichloroethyl sulfide, the inhibition by divinyl sulfone increases with increasing phosphate concentration from 0 to 0.05 M.

To eliminate the possibility that the increased sensitivity of urease to divinyl sulfone was due to the presence of potassium in the buffer rather than phosphate, urease was exposed to 0.002 M divinyl sulfone for 1 hour in 0.025 M phosphate buffer and in 0.025 M potassium chloride solution adjusted to pH 8.5 with potassium hydroxide. Inactivation of only 20 per cent occurred in the potassium chloride solution, compared to 50 per cent in the presence of phosphate.

TABLE II
Inhibitory Effectiveness of Various Substances in Presence of Phosphate

Compound	Concentration	Time	Per cent inactivation of urease	
			In NaOH, pH 8-8.5	In 0.025 M phosphate, pH 8-8.5
	M	min.		
Divinyl sulfone.....	2×10^{-3}	60	16	65
Benzoquinone.....	2.5×10^{-4}	60	37	70
Menadione.....	5×10^{-4}	30	44	34
Iodine.....	3×10^{-6}	15	45	40

To determine whether increased inhibitory effectiveness in the presence of phosphate was a characteristic peculiar to divinyl sulfone or whether it was common to other poisons, the influence of phosphate on the inactivation of urease by three other inhibitory agents was determined. The amounts of inactivation produced by benzoquinone, menadione (2-methyl-1,4-naphthoquinone), and iodine with and without 0.025 M phosphate at pH 8 to 8.5 were measured and are shown in Table II. Increased inhibitory effectiveness in the presence of phosphate was observed with benzoquinone but not with menadione or iodine.⁴

DISCUSSION

It was observed with both the chloroethyl sulfides and divinyl sulfone that inactivation of urease increased markedly with increase of pH from

⁴ Inactivation by menadione and iodine was partially reversible by cysteine, but no reversal of the effects of any of the other agents studied could be obtained with cysteine, cyanide, or change of pH.

6 to 8. This behavior appears to be congruous with the characteristics of alkylation of sulfhydryl groups, which is dependent on dissociation of the sulfhydryl and it is probable that the recognized essential *b* sulfhydryl groups of the enzyme were involved by each of the alkylating compounds.

The observation that β -chloroethyl- β' -hydroxyethyl sulfide caused approximately half as much inhibition as the dichloroethyl sulfide indicates that the effectiveness in producing inactivation was proportional to the alkylating potentialities of these compounds and was not influenced by special properties such as the presence of two reactive radicals in a single molecule, which might lead to cross-link or ring formation.

The influence of phosphate on the inactivation of urease appears complicated because of the completely opposite effects observed with dichloroethyl sulfide and with divinyl sulfone. With dichloroethyl sulfide the decreased inactivation found in the presence of phosphate is probably explained by the fact that phosphate, like hydroxyl, reacts with dichloroethyl sulfide in competition with the enzyme and provides it partial protection. On the other hand, the increased inactivation by divinyl sulfone, which was noted in the presence of phosphate, does not seem explainable by any known effect of phosphate on this compound, but must be attributable to some influence of phosphate on the enzyme, enhancing its susceptibility to inhibition. Whatever the mechanism of this enhancement, it apparently was not related to the state of activation of the enzyme, nor was it an effect observable exclusively with divinyl sulfone, since increased inactivation was also found with benzoquinone in the presence of phosphate. It seems possible that this special influence of phosphate may be dependent on the involvement of other groups than sulfhydryl in the enzyme, since increase in susceptibility was not found with the more specific sulfhydryl reactors, iodine and menadione. It is also possible that the *b* sulfhydryl groups, which are believed to be less accessible than the unessential *a* sulfhydryl groups to alkylation by certain substances such as iodoacetic acid, may similarly be less accessible to divinyl sulfone and benzoquinone, but be rendered more readily accessible by the presence of phosphate.

It is apparent from observation of the effectiveness of these relatively simple variables in modifying the response of a single enzyme to inactivation by alkylating compounds that enzymes in tissue are probably under influences affecting their susceptibility in a far more complex and significant manner. However, by gaining knowledge of some of these factors, ways to modify tissue responses to alkylating agents may become apparent.

SUMMARY

Investigation was made of some of the factors influencing the inactivation of a sulfhydryl enzyme by several alkylating agents, analogous to the

enzyme inhibition believed to occur in bis- β -chloroethyl sulfide tissue poisoning. Urease was used for the purposes of this study to typify sulfhydryl-dependent enzyme behavior.

It was determined that inactivation of urease by 0.001 and 0.002 M bis- β -chloroethyl sulfide increases markedly with increase of pH from 6 to 8, and also increases with decrease in phosphate concentration when the pH is maintained constant.

A similar relationship of inactivation effectiveness to pH was found with β -chloroethyl- β' -hydroxyethyl sulfide and divinyl sulfone. The mono-chloroethyl sulfide was approximately one-half as effective an inhibitor as the dichloro compound.

With divinyl sulfone an increase in urease inhibition was observed with increasing phosphate concentration from 0 to 0.05 M at pH 8 to 8.5. This effect did not appear to be related to potassium concentration. A similar enhanced toxicity toward urease in the presence of phosphate was observed with benzoquinone, but not with iodine or 2-methyl-1,4-naphthoquinone.

The influence of pH was considered to be related to the dissociation characteristics and reactivity of the sulfhydryl group. It appeared probable that the protective action of phosphate in the case of dichloroethyl sulfide poisoning was due to a competitive reaction of phosphate with dichloroethyl sulfide, while there seemed to be no obvious explanation for the contrary effect observed with divinyl sulfone and benzoquinone.

The authors wish to acknowledge the valuable technical assistance of Helen E. Pentz.

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MEASUREMENT OF THE REACTION RATE OF BIS- β -CHLOROETHYL SULFIDE IN AQUEOUS MEDIA*

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Study of the reactions of bis- β -chloroethyl sulfide (hereafter referred to as H) in biological systems has been complicated by the fact that in aqueous media other substances, such as β -chloroethyl- β' -hydroxyethyl sulfide (semi-H), sulfonium salts, and thiodiglycol, are formed and these substances have different physical and physiological properties from the original compound. To study the behavior of the H itself necessitates an analytical method of sufficient specificity to distinguish H from its reaction products and from normal components of biological systems. Methods based on measurement of hydrogen ion or chloride liberation, oxidative titration as by hypochlorite or bromine, or measurement of the amount of reaction with thiosulfate or with available color-producing reagents are not sufficiently specific, since several of the hydrolysis products of H also exhibit in varying degrees the reactions upon which these tests are based. However, by utilizing a method of H determination based on organic solvent extraction and reaction with dichloramine-T previously reported,¹ it has been possible to measure directly the rate of reaction of H in various aqueous media, including blood.²

Methods

The change in concentration of H during hydrolysis in water or saline at different temperatures was measured as follows:

5, 10, or 20 mg. of H were added to 100 ml. of aqueous solution at the desired temperature and shaken vigorously for 25 seconds to effect solution. At frequent intervals 5 ml. samples were withdrawn, added to 5 ml. of cyclohexane³ (du Pont or Eastman Kodak Company, pure) in 50 ml. test-tubes, and shaken immediately for 30 seconds to extract the H remaining unhydrolyzed. By this procedure the H is quantitatively extracted from

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

¹ Kinsey, V. E., and Grant, W. M., *Ind. and Eng. Chem., Anal. Ed.*, in press.

² Measurements made in tissue will be reported (*J. Clin. Invest.*).

³ Purified kerosene (Insecti-Sol) also has the desired solvent properties and may be used in place of cyclohexane or mixed with it.

the aqueous solution into a solvent in which it is stable and the H is effectively separated from semi-H and sulfonium salts which remain in aqueous solution. 1 ml. portions of the cyclohexane layers were then analyzed for H at seven different times during hydrolysis under each set of conditions of temperature and salt concentration.

For determination of the rate of reaction of H in rabbit (albino) blood, 10 mg. of H were added to 50 ml. of heparinized blood and dissolved by rapid shaking for 45 seconds. At intervals thereafter, 5 ml. samples were shaken vigorously for 2 minutes in glass-stoppered test-tubes with 5 ml. of a 4:1 mixture of cyclohexane and purified kerosene. The emulsion was separated into two layers by brief centrifuging and the amount of H extracted was determined by the analytical method already described.

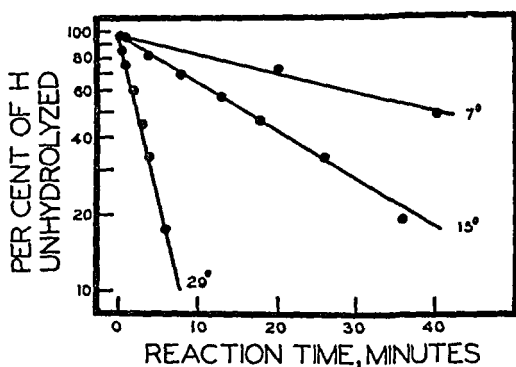


FIG. 1. Hydrolysis of bis- β -chloroethyl sulfide (H) in water at several temperatures, showing the linear decrease of the proportion of H remaining unhydrolyzed with increasing time of reaction.

This procedure was found to give consistent maximal recoveries of H from blood.

Results

The half life of H, as an expression of the rate of reaction, was conveniently determined for each temperature and salt concentration investigated from a plot of the logarithm of H concentration against the time of hydrolysis. Several examples of the results obtained are given in Fig. 1. No difference in rate was observed with different starting concentrations, provided all the H was in solution. The relationship between temperature and the half life of H in water is shown in Fig. 2. It appears from this line that the logarithm of the half life of H in water varies inversely with temperature and that the temperature coefficient is approximately 3.8.

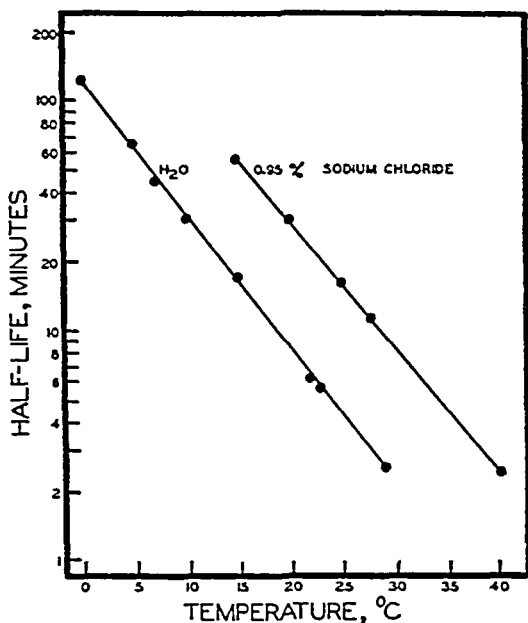


FIG. 2. Relationship between the temperature and half life of bis- β -chloroethyl sulfide in water and in 0.95 per cent sodium chloride solution.

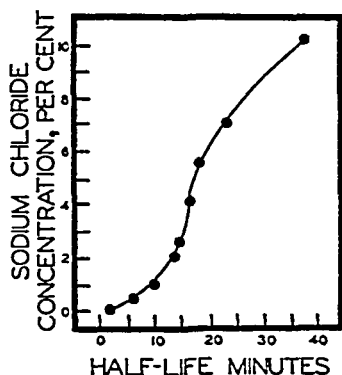


FIG. 3. Relationship of the half life of bis- β -chloroethyl sulfide to sodium chloride concentration at 29°.

The effect of 0.95 per cent sodium chloride on the temperature-rate relationship is also shown in Fig. 2. In this instance, the temperature coefficient is 3.6, similar to that for water solution.

A series of rate determinations made at a constant temperature of 29°, but with different sodium chloride concentrations, is represented in Fig. 3, in which the half life of H is plotted against salt concentration. Up to 3.0 per cent concentration, slowing of hydrolysis of H varies as the square root of the salt concentration. Cohen and Harris⁴ have demonstrated that this relationship of salt concentration applies similarly to slowing of acid formation by hydrolyzing H.

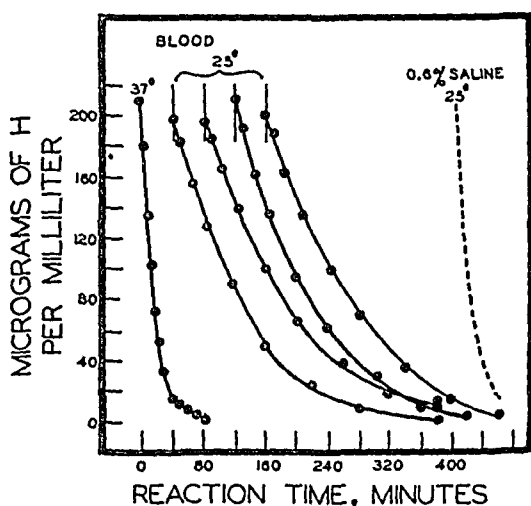


FIG. 4. Curves showing the decrease in concentration of bis- β -chloroethyl sulfide (H) with time of reaction in several samples of rabbit blood at 37° and 25° and in 0.6 per cent saline solution at 25° for comparison.

The values for the half life of H in rabbit blood at 25° determined from the curves of Fig. 4 were 68, 69, 70, and 80 minutes. These are in fair agreement with those obtained by Northrop and Herriott,⁵ most of which lay between 50 to 60 with some at 80 minutes. The persistence of H in blood is apparent from a comparison of the half life of 14 minutes at 37° with that of 3.4 minutes for physiologic saline at the same temperature. Comparison of the half life values in blood at 25° and 37° indicates a temperature coefficient essentially the same as that for water solutions of H.

⁴ Personal communication from B. Cohen and J. Harris.

⁵ Personal communication from R. M. Herriott.

SUMMARY

The rate of the first step of reaction of bis- β -chloroethyl sulfide, involving loss of a single chlorine, was measured in water, sodium chloride solutions, and blood at various temperatures. The temperature coefficient in water was found to be 3.8. Slowing of the reaction by sodium chloride was proportional to the square root of the concentration of this salt up to 3.0 per cent. In rabbit blood the reaction rate was approximately a quarter of that found in 0.6 per cent sodium chloride solution.

A SYNTHESIS OF SERINE AND ITS METHYL ESTER*

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Wood and du Vigneaud (1) have described a satisfactory synthesis for serine, starting with the available esters of acrylic acid. They first prepared the dibromopropionate which was converted into β -ethoxy- α -bromopropionic acid; treatment with ammonia and subsequent deethylation yielded *DL*-serine, with an over-all yield of 47 per cent. This procedure is an improvement over that of Schiltz and Carter (2), who employed β -methoxy- α -chloropropionic acid as an intermediate.

The easily prepared halohydrins of the alkyl acrylates would appear to offer a more satisfactory route to the synthesis of serine. Melikoff (3), however, by allowing β -hydroxy- α -chloropropionic acid to react with ammonia at elevated temperatures obtained β -amino- α -hydroxypropionic acid rather than serine. This unexpected result was explained by the preliminary formation of an epoxy intermediate, $\text{CH}_2\text{---CH---COOH}$; this



reacted with ammonia to form the isomer of serine (4).

During the course of the present investigation it has been found that the bromohydrin of methyl acrylate will react with benzylamine to give good yields of the methyl ester of *N*-benzylserine. The benzyl group may be removed by catalytic hydrogenolysis. The synthesis involves three steps, and the yield, based on methyl acrylate, is 63 per cent.

EXPERIMENTAL

A mixture of 17.5 gm. (0.2 mole) of methyl acrylate, 28.0 gm. (0.2 mole) of potassium carbonate, and 400 ml. of distilled water, in a 4 liter beaker, was cooled to 0°. To this was added a cold solution of 32.0 gm. of bromine (0.2 mole) and 57 gm. (0.54 mole) of sodium carbonate in distilled water; the mixture was stirred for an hour at 0°. After neutralization with hydrochloric acid, the mixture was allowed to stand overnight. The methyl β -hydroxy- α -bromopropionate was then extracted with ether and dried over anhydrous sodium sulfate; volatilization of the solvent left an orange-

*Fourth paper on the synthesis of amino acids. For the preceding papers see Hamlin, K. E., and Hartung, W. H., *J. Biol. Chem.*, 145, 349 (1942); Waters, K. L., and Hartung, W. H., *J. Org. Chem.*, 10, 524 (1945); Mattocks, A. M., and Hartung, W. H., *J. Am. Pharm. Assn.*, 35, 18 (1946).

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colored oil, weighing 30.1 gm. (82.5 per cent). Attempts to distil the oil resulted in decomposition. The identity of the product was confirmed by a negative iodoform test and by its quantitative catalytic reduction to methyl β -hydroxypropionate, which on distillation formed a quantitative yield of methyl acrylate.

To a cold solution of 21.4 gm. (0.2 mole) of benzylamine in 200 ml. of dry ether were added with stirring 18.3 gm. (0.1 mole) of methyl β -hydroxy- α -bromopropionate. Benzylamine hydrobromide crystallized immediately; to facilitate its precipitation, the reaction mixture was further cooled. The salt was removed by filtration; after being washed with ether and dried, it weighed 18 gm. (approximately 0.1 mole), indicating substantially quantitative reaction. To the yellow filtrate were added 50 ml. of absolute ethanol containing 0.1 gm. of hydrogen chloride per ml.; on standing in the refrigerator overnight colorless platelets settled out. These were filtered off, washed with ether, and, after drying *in vacuo* over soda lime, weighed 20.8 gm. (85 per cent). The hygroscopic hydrochloride of methyl β -hydroxy- α -benzylaminopropionate may be recrystallized from absolute ethanol. Found, N (Kjeldahl) 5.65 per cent; calculated for $C_{11}H_{15}O_3N \cdot HCl$, N 5.71 per cent. After alkaline saponification N-benzylserine was obtained, melting at 134–135°. Found, N (Kjeldahl) 7.30 and 7.32 per cent; calculated for $C_{10}H_{13}O_3N$, N 7.18 per cent.

In 50 ml. of absolute ethanol was dissolved 0.4 gm. of N-benzylserine, and the compound was hydrogenated with palladium on charcoal according to the method of Iwamoto and Hartung (5). Although hydrogenation proceeded extremely slowly, the hydrogen calculated for removing the benzyl group was taken up in 6 hours, and, after removal of the catalyst, colorless crystals were isolated from the solution, which darkened at 223–224°, the decomposition point recorded by Fischer and Leuchs (6) for serine. The yield of serine in this instance was but 45 per cent, but there is every reason to believe that the yields may be expected to approach the theoretical values by working with larger amounts.

In 100 ml. of absolute ethanol were dissolved 8 gm. (0.083 mole) of the hydrochloride of methyl β -hydroxy- α -benzylaminopropionate. The solution was treated with 3 gm. of palladium catalyst at 200 pounds per sq. in. Hydrogenolysis of the N-benzyl group was complete in 3 hours. The catalyst was filtered off, and the solution, which had a pronounced odor of toluene, was concentrated to about 50 ml. at reduced pressure and slightly elevated temperature; ether was added and the solution chilled overnight. 4.7 gm. (90 per cent) of colorless crystals were obtained. After recrystallization from alcohol, the product melted at 112–114°, which agrees with the melting point reported for the hydrochloride of methyl β -hydroxy- α -aminopropionate (7). Found, N (Kjeldahl) 9.44 and 8.94 per cent; cal-

A. M. MATTOCKS AND W. H. HARTUNG

culated for $C_4H_9O_2N \cdot HCl$, N 9.03 per cent. The yield of methyl β -hydroxy- α -aminopropionate hydrochloride, based on the initial methyl acrylate, was 63.1 per cent. Treatment of the ester hydrochloride with phosphorus and hydriodic acid according to the method of Fischer and Leuchs (6) yielded α -alanine, melting (with decomposition) at 291° . This formation of α -alanine rather than β -alanine is additional evidence for the structure of the final product and the intermediates.

SUMMARY

The methyl ester of serine hydrochloride was prepared in a yield of 63 per cent, in three steps, from methyl acrylate. The synthesis involves the conversion of methyl acrylate into its bromohydrin, the reaction of the bromohydrin with benzylamine to form the N-benzyl derivative of serine, and the catalytic hydrogenolysis of the N-benzyl group.

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COMPOSITION OF NORMAL BONE MARROW IN RABBITS

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Normal bone marrow varies considerably in its composition. There are variations within the same individual, depending upon the location of the marrow, and between individuals because of functional demand and age. This variable composition has been noted frequently. It has been shown that the water content varies linearly inversely with the lipide (1). However, similar correlations between other components of the marrow have not been described. The early work dealing with the constitution of bone marrow has been summarized by Cheng (2) and Huggins *et al.* (3). Many of the investigations have been based upon isolated samples of marrow without consideration of the age and development of the animals.

Various methods have been used for comparing samples of marrow. Most of the investigators failed to include one or more of the constituents in their comparative analyses. In respiration studies (4), comparisons were obtained by calculating the lipide-free dry weight. Huggins *et al.* (3), using centrifuged marrow, found a relatively constant lipide-free solid content. These workers, however, did not apply the method to the analysis of all marrow samples. McCoy and Schultze (5) neglected to include the water content in comparing marrow from normal and anemic rats and calculated some of their results on the basis of the lipide-free dry weight. In this paper the marrow is considered as a unit, and a correlation is shown among all components investigated.

EXPERIMENTAL

Rabbits of different breeds were killed by exsanguination following a light blow on the head. Marrow from various bones, in quantities of from 100 to 250 mg., was transferred to weighed 15 ml. centrifuge tubes. Care was taken to exclude bone spicules. The samples were then dried to a minimum weight at 105–110°. It was found that 4 to 5 hours of drying at 110° gave the most satisfactory results. If heated for longer periods, weight was gained owing to oxidation of the lipides. The lipides were extracted four to five times with 10 ml. portions of 3 parts of 95 per cent ethyl alcohol and 1 part of ether, a minimum of 1 hour being allowed for each extraction. The marrow was broken up with a glass rod and stirred intermittently. The tubes were centrifuged at 1500

R.P.M. and the solvent decanted into 25 × 200 mm. tubes. The solvent was removed by evaporation on a steam bath. The lipide-free residue was dried, weighed, and transferred to digestion tubes with the aid of sulfuric acid and water. The nitrogen content of both the lipide and the residue was determined by the Kjeldahl method. The term "residue" is used in this paper to designate the solids remaining after the extraction of dry bone marrow with alcohol-ether. The composition of the bone marrow was calculated from the data obtained by the above method. The interrelationship of the components was further studied by correlation analyses, with 155 marrow samples from eleven rabbits at ages ranging from 37 days to 2 years.

TABLE I
Composition of Bone Marrow of Rabbit 3 in Per Cent of Total Marrow

Bone marrow	Water	Lipide	Residue	Total nitrogen	Lipide nitrogen
Metatarsals.....	22.2	74.8	3.0	0.45	0.040
Radius.....	26.4	69.1	4.5	0.63	0.059
Tibia, distal.....	27.6	68.3	4.1	0.62	0.066
Ulna.....	28.9	66.6	4.5	0.64	0.075
Tibia, center.....	46.1	44.2	9.7	1.43	0.159
Humerus, distal.....	52.1	34.5	13.4	1.92	0.245
Femur, center.....	52.6	35.9	11.5	1.77	0.184
Tibia, proximal.....	53.6	35.1	11.3	1.76	0.189
Os coxae.....	54.1	32.8	13.1	2.05	0.233
Humérus, center.....	54.8	32.6	12.6	1.94	0.189
Femur, distal.....	55.0	33.5	11.5	1.84	0.207
Vertebrae, lumbar.....	56.3	30.4	13.3	2.14	0.265
Femur, proximal.....	57.3	29.6	13.1	2.10	0.281
Humerus, proximal.....	63.2	22.6	14.2	2.37	0.316
Ribs.....	65.0	19.8	15.2	2.37	0.437

Calculations—The symbols W , L , R , N , and N_L are used to represent the percentage concentrations of water, lipide, residue (lipide-free solids), total nitrogen, and per cent nitrogen in the lipide, respectively. The calculation of the correlation between two variables was adapted from the methods described by Ezekiel (6) and Croxton and Cowden (7). The multiple correlation among W , L , and R was calculated by the method described by Whittaker and Robinson (8) and Fenn and Haege (9).

Results

Examination and plotting of the data on the composition of the marrow showed that a definite relationship existed between the various components. Table I gives the composition of the marrow from one

animal. The marrow samples taken from similar sites in other rabbits were similarly interrelated with deviations due to age. In general, the metatarsal marrow had the lowest water and residue content and the rib marrow the highest. The proximal ends of long bones were higher in water and residue than the distal ends. The metatarsal marrow of rabbits less than 2 months old showed a higher ratio of water to residue, which was inconsistent with all of the other data, and hence was not included in the calculations.

Correlation of Water, Lipide, and Residue—Since $W + L + R = 100$, the composition of the bone marrow can be represented on triangular coordinate paper. Fig. 1 shows the values for the composition of the marrow obtained from the bones of three rabbits of different ages. The line shown gives the best representation of all marrow. It is not, however, the best that could be drawn for the composition of marrow from individual animals. The inactive marrow is represented in the region

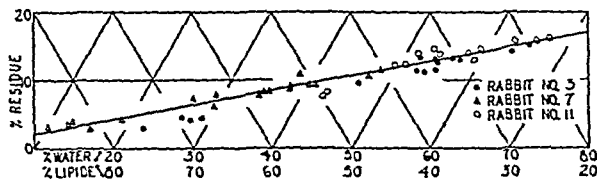


FIG. 1. Composition of normal bone marrow plotted on triangular coordinates. The ages of the animals are Rabbit 3, 3 months; Rabbit 7, 2 years; Rabbit 11, 43 days.

of low water, low residue, and high lipide content. The multiple correlation of these three components is given by the equation $W = 0.001 \pm 0.081L + 4.243 \pm 0.073R$. This equation shows that 4.243 gm. of water are associated with each gm. of residue, and that no water is deposited with the lipide. The simple correlations of these three components are shown in Figs. 2 to 4.

The solid lines in Figs. 2 to 4 indicate the calculated equations for 155 marrow samples from eleven animals, with the standard errors of estimate shown by the broken lines. The effect of age on the variation of the composition of marrow is best shown in Figs. 2 to 4. With increasing age the residue and water decrease and the lipide concentration increases for the marrow in any given site.

Correlation of Total Nitrogen with Water, Lipide, and Residue—The simple correlation of the water, lipide, and residue content of the marrow with its nitrogen content is shown in Table II. The nitrogen content of the marrow is a direct linear function of the water and residue, and an inverse function of the lipide content. The correlation as indicated by the correlation coefficients is approximately equally good for all three regression equations.

In studies on respiration of bone marrow, the results are frequently calculated on the lipid-free dry weight (4). The nitrogen content is multiplied by 6.85 to obtain the lipid-free dry weight. This value was

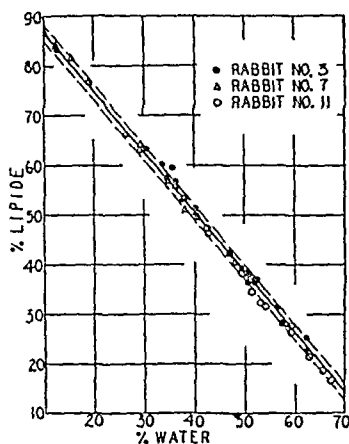


FIG. 2

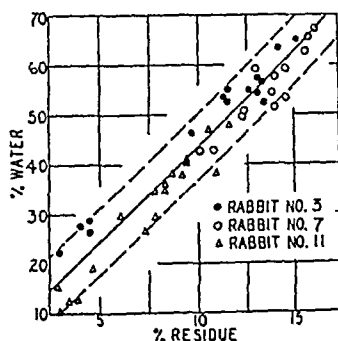


FIG. 3

FIG. 2. Correlation of lipid and water in bone marrow. The solid line corresponds to the equation, $L = 98.98 - 1.211 \pm 0.008W$, with a standard error of estimate of ± 1.50 , and a correlation coefficient of -0.9965 .

FIG. 3. Correlation of water and residue in bone marrow. The solid line corresponds to the equation, $W = 4.83 + 3.85 \pm 0.15R$, with a standard error of estimate of ± 6.80 , and a correlation coefficient of 0.9013 .

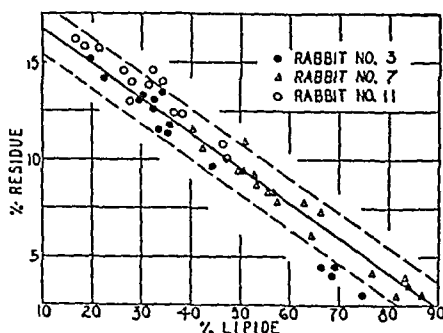


FIG. 4. Correlation of residue and lipid in bone marrow. The solid line corresponds to the equation, $R = 18.52 - 0.180 \pm 0.006L$, with a standard error of estimate of ± 1.31 , and a correlation coefficient of -0.9343 .

determined by Orr and Strickland (10) for pooled samples of marrow of long bones of rabbits. It is not clear whether these workers included the nitrogen content of the lipid fraction. In Table III the results are calculated both as per cent nitrogen in the residue and as total marrow nitrogen percentage of the residue. If the nitrogen extracted by the

alcohol-ether solvent is included, the results are about 0.7 per cent higher. Table III also shows that the nitrogen content of the residue is a function of the water content. The residue of marrow with a high water content has a greater nitrogen concentration than that of low water content. The mean nitrogen concentration of the marrow samples is calculated for each 10 per cent interval of water content. The

TABLE II

Simple Correlation of Water, Lipide, and Residue of Marrow with Its Nitrogen Content (151 Marrow Samples from Eleven Rabbits)

Equation	Standard error of estimate	Correlation coefficient
$W = 6.45 + 25.42 \pm 0.74N$	± 5.31	0.9427
$L = 92.46 - 31.60 \pm 0.72N$	± 5.14	0.9639
$R = 1.084 + 6.18 \pm 0.11N$	± 0.78	0.9780

TABLE III

Nitrogen Content of Lipide-Free Dry Residue

Water in whole marrow	No. of samples	N ₂ in residue, average \pm S.D.*	Total N ₂ of residue, average \pm S.D.*
<i>per cent</i>		<i>per cent</i>	<i>per cent</i>
8-10	3	10.23 \pm 0.308	11.43 \pm 0.158
10-20	8	10.51 \pm 1.376	11.16 \pm 1.358
20-30	16	12.44 \pm 1.555	13.28 \pm 1.586
30-40	21	12.96 \pm 1.155	13.71 \pm 1.190
40-50	24	13.71 \pm 0.850	14.49 \pm 0.908
50-60	47	14.40 \pm 0.746	15.09 \pm 0.818
60-70	27	14.62 \pm 0.641	15.19 \pm 0.655
70-74	4	14.85 \pm 0.289	15.38 \pm 0.252
8-74	150	13.64 \pm 1.502	14.35 \pm 1.473

* Standard deviation calculated by $\sqrt{\frac{\sum x^2 - \bar{x}^2 n}{n-1}}$

difference between the adjacent groups may be due to chance, but the total trend is significant.

A comparison of the standard error of estimate of the regression of residue on nitrogen, $R = 1.084 + 6.18N$, with the standard deviation of the percentage of nitrogen in the residue shown in Table III indicates that a more reliable estimate of the lipide-free solids could be obtained by the use of this equation than by the method commonly used for this purpose (4, 10).

Correlation of Lipide Nitrogen with Lipide—The regression of the lipide on the log of the lipide nitrogen was calculated after multiplying the latter by 100, as this makes the equation easier to use. This regres-

sion is illustrated in Fig. 5. The effect of age on the change of the composition of marrow from the same locations is again shown. As the animal ages, the lipid concentration of the marrow increases, but the lipid nitrogen decreases. The most significant change is that associated with activity of marrow. The lipid nitrogen increases on a logarithmic scale with increased activity. Thus, a relatively larger amount of nitrogen, extracted by alcohol-ether, is found in active marrow.

Lipide-Free Basis—The residue and nitrogen content of the marrow representing 150 samples averaged 19.01 ± 2.768 and 2.71 ± 0.338 per cent, respectively. The marrow samples of greater activity show a

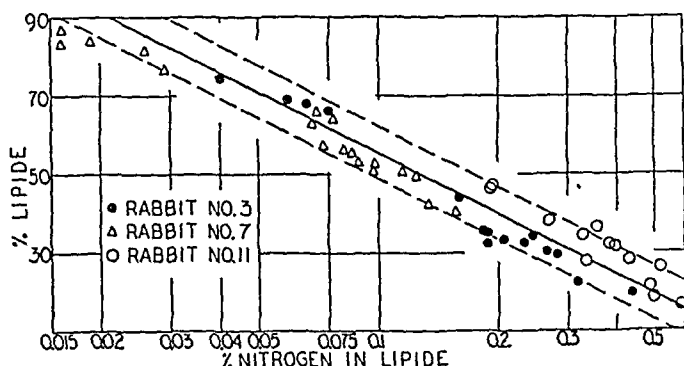


FIG. 5. Correlation of lipid and the log of the lipid nitrogen in bone marrow. The solid line corresponds to the equation, $L = 107.19 - 51.60 \pm 1.60 \log 100N_L$, with a standard error of estimate of ± 6.82 , and a correlation coefficient of -0.9355 .

trend towards a higher residue and nitrogen content within an individual animal, but when the marrow samples of similar activity from different animals are grouped this trend is lost.

DISCUSSION

Warren (4) has shown that the activity of bone marrow is a function of the lipid-free dry weight. From the results of the correlation of the various components, it may be stated that the activity of marrow is a direct function of the nitrogen, residue, water, and log of the lipid nitrogen content, and an inverse function of the lipid concentration. It is difficult to divide the marrow into active and inactive, since all degrees of activity may be found.

With the composition of marrow as a criterion of its activity, it is apparent that there are variations in activity at different points of the long bones. The distal ends are less active than the proximal. This is most pronounced in the tibia, but also holds true for the femur and the humerus. In the last two bones, the diaphyseal marrow shows usually

less activity than the epiphyseal. The ulna marrow is slightly more active than that of the radius, and the activity of both approximate that of the distal end of the tibia. As the age of the animal increases, there is a decrease in the activity of all marrow. This may be compensated, in part, by an increase in the total amount of marrow present (11).

A comparison of marrow on the lipid-free basis shows a relatively constant residue and water composition. This is due to the fact that most of the water is associated with the residue, and very little with the lipid. Such comparisons tend to make all marrow appear to be of the same composition, and "fundamental changes in the composition of animals coincident with growth and aging may be obscured... especially if "fat-free" implies... freedom from all lipid substances" (12). The "essential lipides" certainly have a positive rôle in the function of marrow. If the nitrogen content of the lipid may be considered as an index of the essential lipides present, these components are present in a relatively higher concentration in the active marrow.

SUMMARY

There is a direct linear relationship between the water and residue (lipid-free solids) and an inverse linear correlation between these two components and the lipid content of the marrow.

The total nitrogen concentration of the marrow varies directly with the residue and water and inversely with the lipid.

The log of the lipid nitrogen (extracted with alcohol-ether) is inversely proportional to the lipid concentration.

With advancing age there is a decrease in the water, residue, total nitrogen, and lipid nitrogen and an increase in the lipid concentration of normal bone marrow.

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PYRUVIC ACID IN EXERCISING DEPANCREATIZED DOGS AND DIABETIC PATIENTS*

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(Received for publication, April 8, 1946)

Two apparently contradictory groups of evidence exist in regard to the utilization of carbohydrate in diabetes. If the accumulation of lactic or pyruvic acid is regarded as an indicator of carbohydrate metabolism, then ingested carbohydrate is not metabolized by non-nervous tissues of the resting depancreatized dog (1, 2) or by the diabetic human patient (3, 4), while the exercising diabetic organism, whether canine (5) or human (6), is able to use carbohydrate. The present report deals with a reinvestigation of this problem and includes lactic and pyruvic acid studies in the depancreatized dog and the diabetic patient after the administration of carbohydrate, during exercise, and a combination of the two.

Method

Both canine and human subjects were used for these experiments. Four dogs were depancreatized and maintained in excellent condition, from 4 to 10 months, with 15 units of crystalline insulin, 60 gm. of glucose, and 200 gm. of ground horse meat twice daily, as well as choline chloride, thiamine, riboflavin, niacin, calcium pantothenate, pyridoxine, and vitamins A, D, C, and K thrice weekly.¹ From one to five observations was made on each of the animals after a period of 36 hours, during which they received neither food nor insulin. The animals were anesthetized with pentobarbital and subjected to exercise of their hind legs by the use of an inductorium in parallel with a metronome.

After control samples of arterial blood were collected, the dogs were exercised moderately for 15 minutes and then more strenuously for another 15 minutes. The degree of exercise was the same in all animals. Blood samples were collected after each 15 minute period as well as at $\frac{1}{2}$, 1, and 2 hour intervals after exercise was completed. Observations on two of the four

*We gratefully acknowledge the financial aid extended by the New York Diabetes Association.

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¹The insulin was generously supplied by Dr. F. B. Peck of Eli Lilly and Company, vitamins C and K by Dr. M. L. Tainter of the Winthrop Chemical Company, Inc., and all other vitamins used in this study by Dr. Y. SubbaRow of the Lederle Laboratories, Inc.

animals were made both before and after pancreatectomy. A fifth animal was used to obtain additional normal control values. For two of the depancreatized dogs, glucose tolerance tests with and without superimposed exercise were made.

Three human subjects with severe diabetes, probably of pancreatic origin, were examined under three different conditions: exercise without food, food without exercise, both food and exercise. All three were studied about 16 hours after their last dose of insulin and before they had their morning dose on the test day. Two of these patients were in the Albany Hospital for regulation and were receiving only crystalline insulin; in the third patient, subject C, the diabetes was controlled with protamine zinc insulin. The food ingested was the regular morning meal and the exercise consisted of a step up, repeated thirty times per minute for a total of 15 minutes. Similar exercise was done by two normal controls in the postabsorptive state. Venous blood samples were drawn before and after breakfast, before and

TABLE I

Blood Pyruvate (Mg. Per Cent) with Exercise before and after Pancreatectomy
Values after "control" represent differences from that figure.

Total time, min.....	30		60		120	Condition
Time intervals, min....	15	15	30	30	60	
Control	Exercise		Rest			
0.84	+0.43	+1.26	-0.50	-0.21	-0.11	Normal
1.58	+0.46	+1.26	0.00	-0.64	-0.32	Depancreatized

after exercise, and at hourly intervals for 2 hours after the exercise. Pyruvic acid (7), lactic acid (8), and glucose (9) were determined on each sample of blood drawn.

Results

In Tables I to IV are presented typical figures for pyruvic acid in one dog under a variety of conditions rather than the average values for all the animals. Lactic acid to pyruvic acid ratios are not given, as they increase with exercise and confirm similar figures in the literature (10). Blood glucose and lactate values are included only when they are pertinent. The effect of exercise upon blood pyruvate in the same dog both before and after pancreatectomy is shown in Table I. For Table I only, all values after the control figure are changes from the original level. The increases are comparable and the rise of pyruvic acid with exercise was practically the same before and after excision of the pancreas. These observations are typical of three before and three after pancreatectomy in this dog and are in agree-

ment with a similar pair of observations on a second dog as well as of seven experiments on three additional exercising depancreatized dogs and a normal control.

Table II gives average values of blood pyruvate on a single diabetic dog. A rise in pyruvic acid does not occur after the injection of glucose in the resting animal, but the increase is significant with exercise alone and is slightly larger when exercise is accompanied by glucose injection, either 2 gm. or 1.5 gm. per kilo. A similar difference between exercise alone (average of four observations) and glucose and exercise (one observation) was noted in another depancreatized dog for which the data are not given.

TABLE II
Average Blood Pyruvate (Mg. Per Cent) in One Depancreatized Dog Given Glucose with and without Exercise and Exercise Alone

	Total time							No. of observations	Remarks
	1 hr.				2 hrs.		3 hrs.		
	Time intervals								
	15 min.	15 min.	15 min.	15 min.	30 min.	30 min.	60 min.		
Control									
1.38	1.38	1.13		1.00	1.13			1	2 gm. glucose per kilo
			Exercise						
0.95	1.29	1.28	1.65	2.41	1.44	1.31		2	2 gm. glucose per kilo + exercise
1.32	1.58	1.52	1.68	2.68	1.50	1.12	1.24	1	1.5 gm. glucose per kilo + exercise
		Control							
		1.11	1.90	2.35	1.47	1.10	1.46	4	Exercise

Simultaneous changes in blood glucose for the same dog reported in Table II are found in Table III. Though exercise appears to have no accelerating effect upon the fall of blood sugar towards the original value, it must be remembered that exercise alone is followed by a gradual rise of approximately 100 mg. per cent in blood sugar level. In a total of four observations on two dogs (one reported in Table III), the administration of glucose, 2 gm. per kilo, resulted in a utilization with exercise similar to that at rest.

Blood lactate values in general followed the same pattern as did blood pyruvate (Table IV), but the changes were greater. The injection of glucose in the resting animal caused a slight increase (2.8 mg. per cent) in the

TABLE III

Average Blood Sugar (Mg. Per Cent) in One Depancreatized Dog Given Glucose with and without Exercise and Exercise Alone

	Total time							No. of observations	Remarks
	1 hr.				2 hrs.		3 hrs.		
	Time intervals								
	15 min.	15 min.	15 min.	15 min.	30 min.	30 min.	60 min.		
Control									
462	1030	885		695	680			1	2 gm. glucose per kilo
			Exercise					2	2 gm. glucose per kilo + exercise
370	841	718	603	524	497	441			
388	656	610	520	430	432	432	392	1	1.5 gm. glucose per kilo + exercise
		Control							
		383	419	392	485	454	434	3	Exercise

TABLE IV

Average Blood Lactate (Mg. Per Cent) in One Depancreatized Dog Given Glucose with and without Exercise and Exercise Alone

	Total time							No. of observations	Remarks
	1 hr.				2 hrs.		3 hrs.		
	Time intervals								
	15 min.	15 min.	15 min.	15 min.	30 min.	30 min.	60 min.		
Control									
10.7	12.6	13.5		11.9	6.5			1	2 gm. glucose per kilo
			Exercise					2	2 gm. glucose per kilo + exercise
3.2	6.9	6.4	7.5	21.3	6.3	5.3			
5.4	6.3	7.3	10.5	19.6	8.6	5.8	5.5	1	1.5 gm. glucose per kilo + exercise
		Control							
		5.6	10.4	13.1	8.6	7.0	6.4	4	Exercise

lactate level, exercise alone caused a much larger rise (7.5 mg. per cent), and exercise accompanied by the injection of sugar was followed by a still greater formation of this metabolite (13.1 and 14.2 mg. per cent). Such a result,

however, may be attributed to a difference in the rate and efficacy of the exercise.

The results for blood pyruvate obtained on two of the three human subjects are presented in Table V. It will be seen that breakfast was followed by only small variations from the control value. The increase with exercise alone is significant and approximates the normal. The rise of pyruvate with breakfast and exercise is of the same magnitude as that with exercise alone in one patient, subject H, but larger in the other one, subject C. The values obtained on the third patient were similar.

TABLE V

Blood Pyruvate (Mg. Per Cent) with Ingested Carbohydrate, Ingested Carbohydrate and Exercise, and Exercise Alone

Subject		Total time					Remarks
		1 hr.		2 hrs.		3 hrs.	
		Time intervals					
		30 min.	30 min.	30 min.	30 min.	60 min.	
	Control	Breakfast					
H	1.73	1.58	1.58		1.42	1.68	Breakfast
C	0.94	1.05	1.30		0.89	0.79	
			Exercise				
H	1.73	1.68	2.10		1.89	1.52	" + exercise
C	0.68	1.00	2.15		1.68	1.36	
		Control					
H		1.31	1.63	1.42			Exercise
C		0.47	1.47	0.94			

DISCUSSION

Pyruvic acid can be formed by exercising muscle and subsequently removed from the blood stream just as readily in the depancreatized as in the normal animal, a result in agreement with previous results on lactic acid (5, 6). In contrast to these findings, we observe that the administration of glucose does not produce a significant increase in blood pyruvate in the depancreatized dog unless accompanied by exercise, indicating that muscular work facilitates the formation of pyruvate and lactate, probably from muscle glycogen. The observations also reveal that glucose, when injected into the diabetic animal, is withdrawn no more rapidly by the tissues from the blood in exercise than at rest. This result is surprising in view of the lowered insulin requirement of diabetics in exercise.

In general the changes in the human patients were the same as in the

experimental animals, even though the patients were not completely diabetic. They showed little or no pyruvic acid increase following the ingestion of a breakfast high in carbohydrate. One of the two patients (Table V) exhibited a rise that was greater with both breakfast and exercise than with exercise alone.

The failure of pyruvic and lactic acids to accumulate in the blood stream of the diabetic organism after carbohydrate administration, considered in conjunction with the rise taking place in exercise, suggests that only the initial phosphorylation of glucose occurring in the hexokinase reaction requires insulin (11, 12). Apparently other intermediary steps of the Embden-Meyerhof scheme do not depend on that hormone.

The arterial blood drawn from the dogs is an indicator of the combined changes occurring within the body. We know that at least two organs, the liver and the heart, absorb pyruvic acid from the blood stream.² Therefore, if the venous blood had been drawn directly from the exercising muscles, even greater accumulations of pyruvate than are reported here might have been expected. In the human subjects in whom blood was collected from the antebrachial vein, the average of the body, as found in the artery, might have been further diminished by the absorption of pyruvic acid in the resting tissues.

Acetoacetic acid, if present, is determined by the Friedemann and Haugen (7) method for pyruvate, with xylene for extraction. In our dogs, ketosis was probably an insignificant phenomenon. For example, three control values for one normal dog were 1.20, 0.84, 0.84 mg. per cent and for the same dog after pancreatectomy, 0.89, 1.89, 1.58 mg. per cent. We believe that the instances in which the blood pyruvate was elevated in the control samples are due to an anoxia resulting from an excessive though transitory barbiturate depression.

SUMMARY

1. Exercising depancreatized dogs form pyruvic acid as readily as do normal animals, but injected glucose fails to produce the normal rise of pyruvic acid in the resting diabetic animal. Injected glucose, moreover, does not disappear more rapidly in the exercising than in the resting depancreatized dog. In general the results in human patients with diabetes are the same as in depancreatized dogs.

2. All the results in diabetic dogs and men presented in this paper support the observations, *in vitro*, of Colowick, Cori, and Price that insulin is active in facilitating the hexokinase reaction and in addition suggest that the other

²Randles, F. S., Himwich, W. A., Homburger, E., and Himwich, H. E., *Am. Heart J.*, in press. Himwich, W. A., and Himwich, H. E., *Am. J. Physiol.*, in press.

intermediary reactions of the Embden-Meyerhof scheme are independent of that hormone.

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THE EFFECT OF A SUSTAINED HYPERCHOLESTEROLEMIA ON THE LIPIDES AND PROTEINS IN THE PLASMA OF THE RABBIT

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In the rabbit hyperlipemia is readily induced by the feeding of cholesterol and may reach enormous proportions. The literature reporting the changes produced in rabbits by cholesterol feeding is extensive. In only a few investigations, however, and with only a small number of animals, was the effect on the plasma proteins studied. Schwarz and Lichtenberg (1), in a single rabbit, found no change in the total protein over a period of 92 days in which the plasma cholesterol rose to 1764 mg. but observed a fall in the albumin to globulin ratio from 2.5 to 1.2. Using a different technique, Mauriac, Servantie, and Demenier (2) made a similar observation. They induced hyperlipemia in a rabbit by alternating cholesterol injections with injections of uranium nitrate for 24 days and found no change in the total protein but a fall in the albumin to globulin ratio coincident with the rise in cholesterol. In both these studies cholesterol was the only lipide determined.

The experiments reported in this paper extend the above studies to a larger series of animals and to analyses of other lipide fractions besides the free and total cholesterol. The hypercholesterolemia was produced by feeding lanolin (cholesterol ester) to three rabbits and pure cholesterol to four rabbits for prolonged periods (88 to 215 days).

Methods

Adult white rabbits, weighing between 3 and 4 kilos, were used. All were kept in individual cages and were maintained on a stock diet of equal parts of Purina rabbit chow and rolled barley.

The animals were divided into three groups. The first group, consisting of three male rabbits, on alternate days was fed a supplement of 15 gm. of anhydrous lanolin (Merck) in 10 cc. of cottonseed oil. The lanolin was warmed in the oil until liquid and was then thoroughly mixed with the grain. This amount of lanolin contains approximately 2 gm. of cholesterol as the ester, which is equivalent to 1 gm. of cholesterol per day. The

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second group, consisting of four rabbits (two males and two females), was fed a supplement of pure cholesterol (Eastman) at a level of 1 gm. per day. The cholesterol was dissolved in hot 95 per cent alcohol and the hot solution was poured over a portion of the grain and then allowed to evaporate. During most of the time 2 gm. of cholesterol were fed on alternate days, but toward the end of the experiments 1 gm. was fed each day. The third group, consisting of two rabbits (one male and one female), was given no cholesterol, but was maintained on the stock diet as a control group.

The experimental animals were kept at first on the basal diet without supplement for about a month, during which time several blood samples were analyzed to establish normal values. In the morning, before feeding, blood was collected from the marginal vein of the ear. A small incision was made in the vein and after the first few drops had been discarded the blood was collected in a paraffin-lined tube containing a few mg. of heparin.

The blood was centrifuged immediately and the following substances were determined in the plasma: total cholesterol, free cholesterol, total fatty acids, lipid phosphorus, total protein, albumin, globulin (by difference), fibrinogen, and non-protein nitrogen. In some of the animals cell volumes and red blood cell counts also were determined.

The total cholesterol and free cholesterol were determined by the method of Schoenheimer and Sperry (3). Readings were made in an electrophotometer. Total fatty acids were determined by the method of Stoddard and Drury (4) as modified by Man and Gildea (5). Lipid phosphorus was determined by the Man and Peters (6) adaptation of the Fiske-Subbarow (7) method. The plasma proteins were separated by precipitation with sodium sulfate and the nitrogen of the fractions was determined by a micro-Kjeldahl procedure (8).

Cell volumes were determined with Wintrobe hematocrit tubes centrifuged at 2500 R.P.M. for 30 minutes. Red blood cell counts were made with Thoma diluting pipettes in a Levy-Hausser counting chamber on heparinized blood diluted with Hayem's solution.

DISCUSSION

The results of our experiments are summarized in Figs. 1 to 7. No consistent differences are shown between the animals fed lanolin (cholesterol ester) and those fed pure cholesterol. In all cases there was a rapid increase in the lipides of the plasma. For total cholesterol both the greatest increase, from a normal average of 30 mg. per 100 cc. to a maximum of 2475 mg. per 100 cc. on the 70th experimental day (Rabbit 10), and the smallest increase, from a normal average of 39 mg. per 100 cc. to a maximum of 852 on the 89th experimental day (Rabbit 8), are shown by animals fed pure cholesterol.

RABBIT 4 ♂

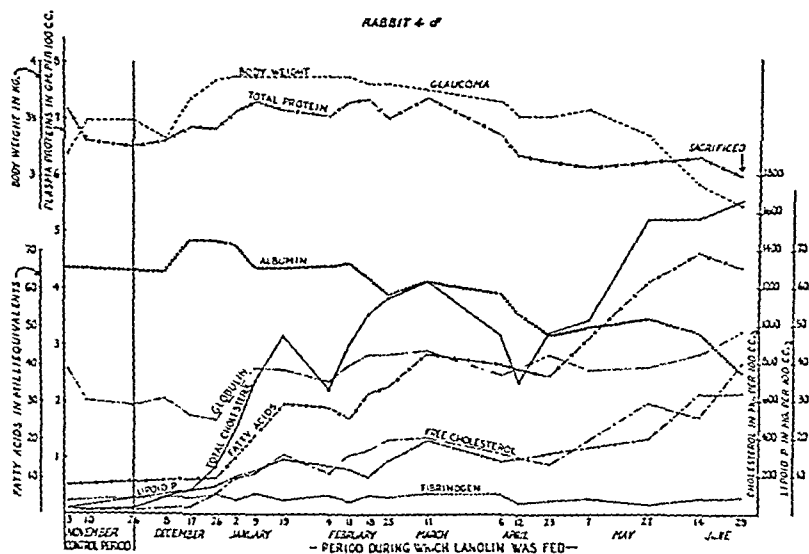


FIG. 1. Changes in proteins and lipides of plasma during the feeding of lanolin

RABBIT 5 ♂

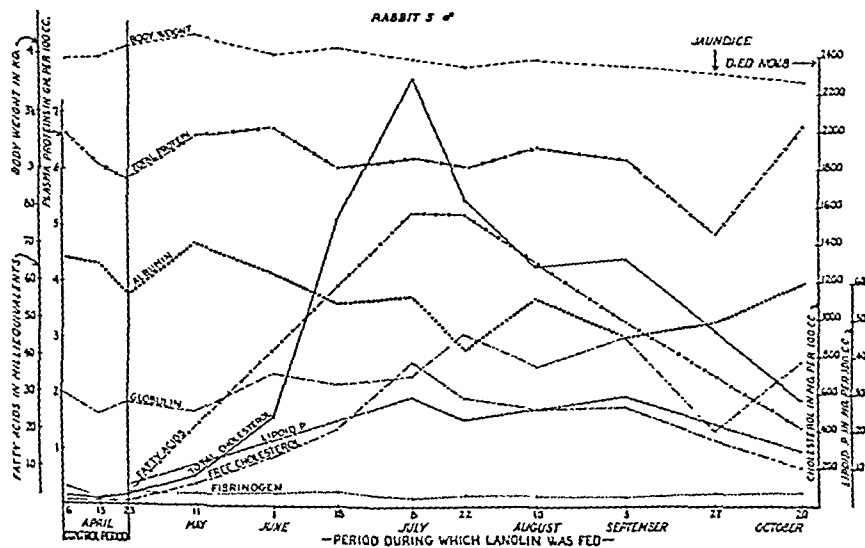


FIG. 2. Changes in proteins and lipides of plasma during the feeding of lanolin

EFFECT OF HYPERCHOLESTEROLEMIA

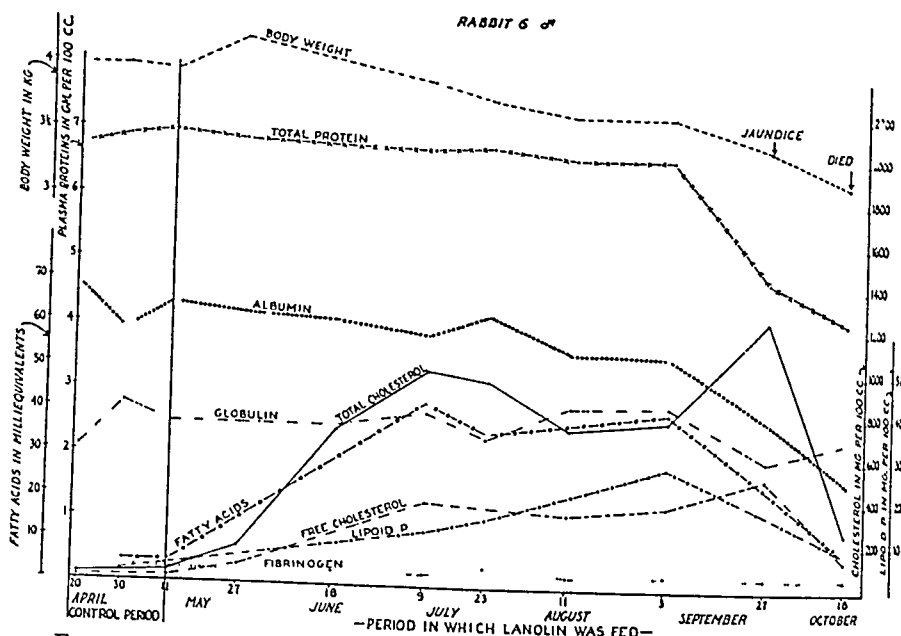


FIG. 3. Changes in proteins and lipides of plasma during the feeding of lanolin

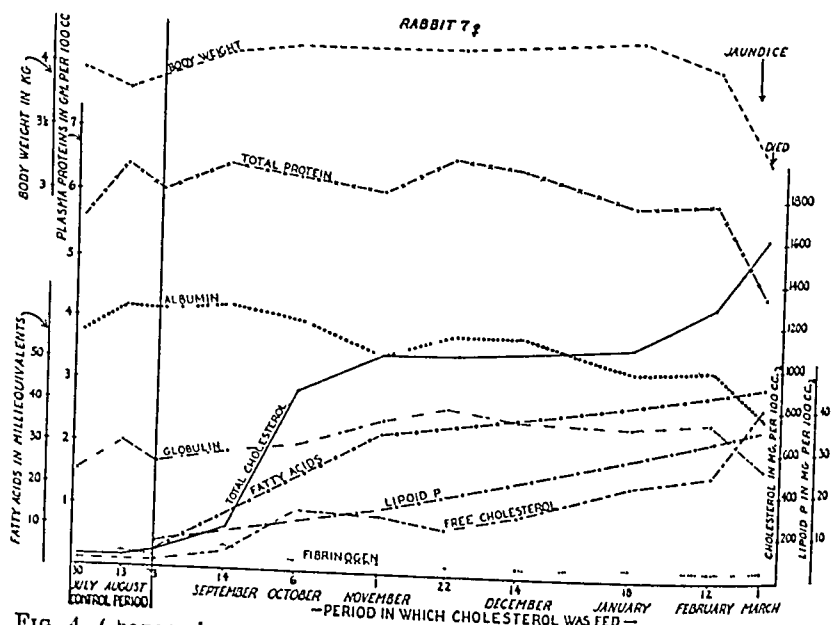


FIG. 4. Changes in proteins and lipides of plasma during the feeding of pure cholesterol.

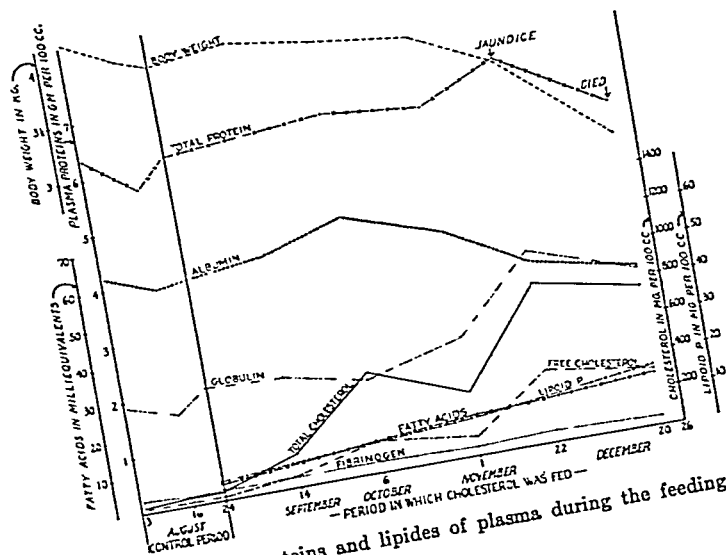


FIG. 5. Changes in proteins and lipides of plasma during the feeding of pure cholesterol.

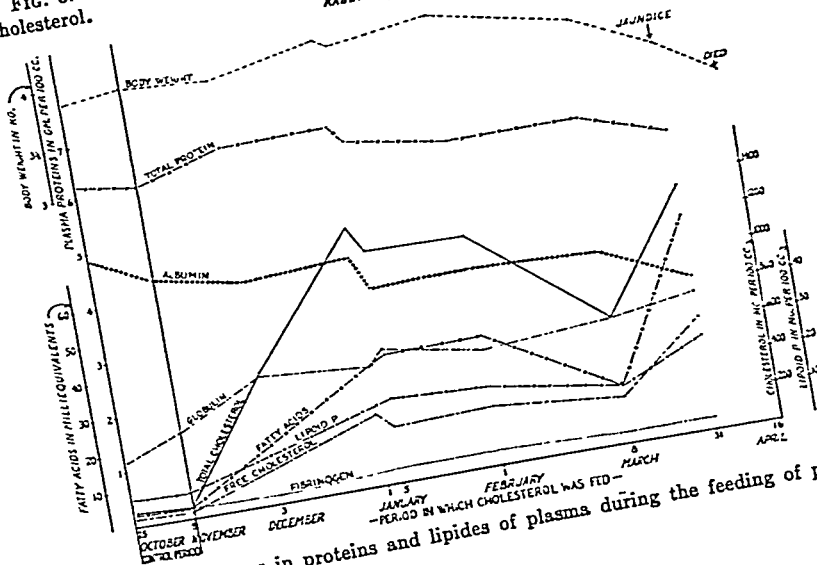


FIG. 6. Changes in proteins and lipides of plasma during the feeding of pure cholesterol.

Turner (9) has shown that atherosclerosis is readily produced by feeding dry cholesterol. However, Weinhouse (10) has concluded on the basis of previous work of Page and Bernhard (11) with that of Weinhouse and Hirsch (12) that feeding fat with cholesterol increases the hypercholesterolemia in rabbits. The same conclusion is implicit in the recent review of Hueper (13). Our work does not support this conclusion, but indicates that hypercholesterolemia, in agreement with the finding of Turner in regard to atherosclerosis, is as readily produced without the use of an oil solvent. It should be pointed out, however, that the discrepancy between

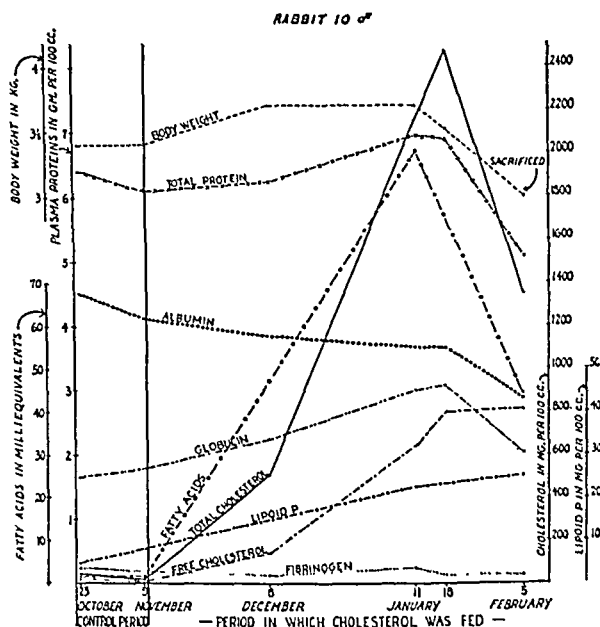


FIG. 7. Changes in proteins and lipides of plasma during the feeding of pure cholesterol.

our finding and the conclusions of Weinhouse and of Hueper may be caused by our use of cholesterol esters when oil was used as a vehicle and the use of free cholesterol when no vehicle was employed.

Two types of cholesterol curves are seen in the graphs: one in which the cholesterol rises rapidly, then levels off for a time, and later rises again (Rabbits 4, 6, 7, and 9); and another in which the cholesterol rises to a peak and then steadily declines until the end of the experiment (Rabbits 5 and 10). Weinhouse and Hirsch (12) reported curves of the latter type after feeding cholesterol to rabbits. They attributed the decline in cholesterol to impairment of the absorption of food as a result of infiltration of lipides into the transporting cells. This conclusion was borne out by a loss of

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weight that paralleled the decline in lipide. Our results do not support this view. In the animal (Rabbit 5) showing the most marked drop in cholesterol, no loss in weight occurred, although the cholesterol fall continued over a period of more than 3 months. If the fall in cholesterol is caused by a diminution in absorption, the effect must be limited chiefly to the lipides. Shreder (14) reported that the two types of curves were caused by sex differences. In his work female rabbits when fed cholesterol showed a rapid rise in blood cholesterol followed by a fall, while the blood cholesterol of cholesterol-fed males continued to rise during the whole period of administration. Upon these findings he based a hypothesis that the lipide regulation in the female animal takes place in a different manner than in the male. However, the two types of curves in our experiments bear no relationship to sex differences. In fact the two cholesterol curves most characteristic of the two types (Figs. 2 and 4) appear in rabbits of the wrong sex to support Shreder's hypothesis.

The average ratio of cholesterol ester to free cholesterol found in twenty-seven samples of normal plasma from nine rabbits was 1.13, with the lowest value for a single sample, 0.5 (Rabbit 4) and the highest value, 1.9 (Rabbit 11). In his recent review, Hueper (13) states that the rise in total cholesterol after feeding cholesterol to rabbits "is not accompanied by any consistent changes in the ratio of free to esterified cholesterol." However, in our experiments the ratio rose in every case during cholesterol feeding and, although, in general, the higher values were associated with higher cholesterol values, this was not always true. In 53 determinations of the cholesterol ester to free cholesterol ratio on cholesterol-fed animals the highest value, 3.7 (Rabbit 7), occurred with a total cholesterol value of 1050 mg. per 100 cc. The highest total plasma cholesterol value found in any of the rabbits, 2475 mg. per 100 cc. (Rabbit 10), occurred with a ratio of 2.1.

In all cases, as the cholesterol increased, the other lipides also increased. The correlation between cholesterol and the other lipide fractions may be closer than appears from the charts, since the fatty acids and phospholipides were determined less frequently than the cholesterol, and some of the fluctuations may have been missed.

The "visible lipemia" which appears in rabbits during cholesterol feeding has attracted the interest of many investigators, because in this species it is impossible to produce a visible alimentary lipemia by feeding neutral fat alone (13, 15). Six of the seven animals used in our experiments showed a visible lipemia that first appeared after 24 to 68 days of cholesterol feeding. The height of the plasma cholesterol at the time the visible lipemia was first observed varied from 447 mg. per 100 cc. in Rabbit 4 to 2155 mg. in Rabbit 10. Rabbit 8 at no time showed a visible lipemia, although the total cholesterol of the plasma rose to 852 mg. per 100 cc., which is considerably

higher than the levels at which a visible lipemia appeared in Rabbits 4, 5, and 9 (447, 480, and 716 mg. per 100 cc., respectively). All the experimental rabbits, with the exception of Rabbit 4, developed jaundice late in the experiment. In several cases the visible lipemia completely or nearly completely disappeared and the plasma became a dirty green when jaundice developed, without appreciable decreases in the plasma cholesterol level.

During the development and maintenance of the lipemia there was no significant change in the total protein. The fibrinogen fluctuated without any definite trend. On the other hand, all the animals showed a slight but consistent fall in the albumin as the experiment progressed and all but one (Rabbit 6) showed a simultaneous increase in the globulin, which was somewhat larger than the decrease in albumin.

Near the end of the experiment, in all the animals except one (Rabbit 5) a marked decline in the albumin was coincident with a decline in the body weight. In three of the animals (Rabbits 4, 5, and 9) the globulin rose just before death; in four (Rabbits 6, 7, 8, and 10) it declined. In all of the animals there was a terminal retention of non-protein nitrogen.

Red blood cell counts and determinations of cell volume were made only a few times and on only a few of the animals. There are sufficient data, however, to indicate that both of these values were subnormal in the latter half of the cholesterol feeding period. The anemia in all cases is shown to be of the macrocytic type, with a volume index greater than 1. These findings are not in agreement with those of Weinhouse and Hirsch (12) who found no significant change either in hemoglobin or in the number of erythrocytes when rabbits were fed cholesterol.

No significant changes in weight were observed until shortly before the end of the experiment, when a decline in weight occurred coincident with loss in appetite. At no time did any of the animals show any evidence of edema.

Schoenheimer (16) states that in his experience a rabbit which has been fed cholesterol for a short time shows a rough coat and a general lack of interest in its surroundings. The apathy of the cholesterol-fed rabbit has been noted also by Versé (17). The animals used in our study all maintained well kept coats throughout the experimental period with the exception of Rabbit 5 which was shedding hair when the experiment began and continued to do so. No change in the disposition or activity of the animals was observed until near the end of the experiment, when they began to refuse food and to consume less water.

Rather large volumes of blood were necessary for the determinations. The greatest total amount drawn was 313 cc. over a period of 214 days in the case of Rabbit 4 and the least, 76 cc. over a period of 88 days in the case of Rabbit 10. It was thought possible that the blood changes found in the

experimental animals might have been caused by the withdrawal of blood. To test this hypothesis two control animals were bled at frequent intervals over a period of 6 months and the same determinations were carried out as with the bloods of the experimental animals. A total of 240 cc. was drawn from one rabbit in 173 days and 234 cc. from the other in 171 days. In one of the animals there was a slight rise in the albumin to globulin ratio. Aside from this, there were no significant changes in either the proteins or the lipides at any time in these two animals.

The only significant influence that the high plasma lipides had on the proteins in our experiments is shown in the fall in the albumin fraction and the rise in the globulin. These changes did not become significant until after the first 2 or 3 months of cholesterol feeding. Mauriac, Servantie, and Demenier (2) reported significant falls in the albumin-globulin ratio in a few days after injections of cholesterol in a rabbit made nephritic with uranium nitrate. Schwarz and Lichtenberg (1) reported significant rises in globulin after 4 weeks in a rabbit fed 2 egg yolks daily. 2 egg yolks per day would furnish from 0.5 to 1.0 gm. per day of cholesterol (18). It would furnish also about 4 gm. per day of phospholipide (19, 20). In our experiments no phospholipide was included in the dietary supplement.

Separations of the protein fractions were made by precipitation with sodium sulfate. It is fair to ask what effect the high plasma lipide had on these separations. It is recognized that, normally, significant amounts of lipide are associated with protein in the plasma. It seems, therefore, that the many attempts to answer this question by removing the lipide from the plasma and comparing protein fractionation before and after this procedure are hardly pertinent. On the other hand, any changes in the protein fractionation that can be demonstrated after additions of lipide to normal plasma would point to the lipide as the causative factor. Went and Faragó (21) prepared emulsions of lecithin in water and added different amounts to 10 cc. portions of serum. After allowing the mixture to stand for $\frac{1}{2}$ hour, they separated the protein fractions by Howe's sodium sulfate method. Of two dog sera one showed no changes and the other showed small reciprocal changes between albumin and globulin at lecithin values near the upper normal limit. At higher values of lecithin the protein fractions returned to the levels of the untreated serum. With the same treatment the serum of a pig showed slight inconclusive changes. Our results seem to indicate that a large increase in the lipides does not of itself affect the precipitation of total globulin with sodium sulfate, because a considerable number of our analyses showed essentially unchanged values for albumin and globulin after the lipemia had become severe.

When the albumin to globulin ratio decreased, the greatest decrease often followed the peak in the lipide values by several weeks. This is par-

ticularly noticeable in Rabbit 5, as seen in Fig. 2. For this reason we believe that the changes in the protein fractions are of a metabolic nature in response to high blood and tissue lipides and are not merely a physicochemical adjustment to these high lipide levels.

SUMMARY

1. Three rabbits were fed cholesterol ester (lanolin) and four rabbits, pure cholesterol, over periods of 88 to 215 days. The plasma was analyzed at intervals for total cholesterol, free cholesterol, total fatty acids, lipoid phosphorus, total protein, albumin, globulin (by difference), fibrinogen, and non-protein nitrogen. In some animals, cell volumes and red blood cell counts also were determined.

2. No consistent differences are shown between the animals fed cholesterol ester and those fed free cholesterol. In every case there was a rapid increase in all the lipides determined. Six of the seven animals showed a visible lipemia. The time of appearance of lipemia could not be correlated with the duration of lipide feeding nor with the concentration of lipide in the plasma. Two types of cholesterol curves were produced: the one in which the cholesterol rises rapidly, then levels off for a time, and finally rises again just before death; the other in which the cholesterol rises to a peak and then steadily declines until the end of the experiment. In the second type, if the decline was caused by diminution in absorption, the effect must have been limited chiefly to the lipides, because there was no loss of weight, although the cholesterol fall continued over a period of months. The two types of curves cannot be correlated with sex.

3. All of the cholesterol-fed rabbits in which red blood cell counts and cell volumes were determined showed an anemia of the macrocytic type.

4. During the development and maintenance of lipemia there was no significant change in the total protein. Fibrinogen fluctuated without any definite trend. A fall in the albumin to globulin ratio became significant after 2 or 3 months of cholesterol feeding and usually followed the peak in the lipide values by several weeks. This lag in the decline in albumin to globulin ratio suggests that the changes in the protein fractions were of a metabolic nature and were not merely physicochemical adjustments to high lipide values.

5. None of the rabbits showed evidence of edema at any time.

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THE PHOSPHATASE ACTIVITY OF HUMAN SPERMATOZOA

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The metabolic activity of human spermatozoa, particularly in relation to the motility of these cells, has been investigated in considerable detail (1-3). It has been shown that the human spermatozoon derives the energy for its motility primarily, if not entirely, from the breakdown of glucose to lactic acid, and that motility proceeds maximally for extended periods under anaerobic rather than aerobic conditions. Indeed, it is doubtful whether oxygen plays any positive rôle in the metabolism of mature human spermatozoa, despite the fact that certain components of the cytochrome respiratory system are present and can be demonstrated by indirect methods (3). The oxygen consumption of the actively motile cells is minute relative to glycolysis and leads only to the production of hydrogen peroxide in amounts sufficient to prove toxic to the cells, as indicated by the failure of motility at oxygen tensions above 20 per cent (3).

The subordination of the reactions of oxidation to those of glycolysis leads to the possibility that phosphate transfer may be of major significance in the metabolic processes of the cell. It was, therefore, thought desirable to investigate the metabolism of human spermatozoa in the presence of those phosphate esters which are of known significance in carbohydrate metabolism in other tissues. The compounds available to us included adenosine triphosphate, adenylic acid (yeast and muscle), the various hexose mono- and diphosphates, and β -glycerophosphate.

EXPERIMENTAL

Methods

The spermatozoa were obtained by low speed centrifugation of pooled specimens of normal human semen. In the preliminary experiments, the packed cells were washed once by resuspension in Ringer's solution and recentrifugation. In later experiments, this washing process was repeated twice. The washed cells were finally suspended in a suitable volume of Ringer's solution, and cell counts and motility determinations were made by methods which have already been described (1). Aliquots of the cell suspension were then incubated at 38° in Warburg manometers for the desired

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length of time, usually 3 or 4 hours, in the presence of the compound under investigation. In most experiments, the gas phase contained 5 per cent CO_2 , in either N_2 or O_2 , as the experiment called for, with sufficient bicarbonate added to the medium to insure a pH of approximately 7.4. Glucose when present was at a concentration of 100 mg. per cent.

Manometric readings were taken at suitable intervals to determine (1) the lactic acid formation under control conditions, (2) the possible production of acids, other than lactic, or of base, by hydrolysis of the phosphate esters, and (3) as an aid in evaluating the possibility of phosphate transfer rather than the liberation of inorganic phosphate. In regard to (3), a similar approach to the problem has been made by Colowick and Kalekar (4). At the close of the experimental period, a fraction of a drop of the vessel contents was usually removed for cell motility determination, and the remaining vessel contents treated with 0.5 cc. of 20 per cent trichloroacetic acid solution and transferred quantitatively to a graduated container for the preparation of the protein-free filtrate, on which subsequent chemical analyses were run.

We have found that trichloroacetic acid does not completely deproteinize the average semen preparation. This effect is more pronounced with fewer washings of the cells; so it is presumably due to adhering seminal fluid protein. Completely protein-free preparations are readily obtained by adding phosphotungstic acid to the trichloroacetic acid preparation, approximately 0.1 cc. of 5 per cent phosphotungstic acid per 10 cc., the final volume being usually sufficient. Any phosphotungstic acid in excess in the filtrate will interfere with subsequent phosphate analyses by producing a small blank color in proportion to the excess of phosphotungstic acid present. This excess will be constant for a given experiment, however, so that differences between two filtrates will be significant, even though the absolute values for phosphate content cannot be determined accurately in the presence of phosphotungstic acid. These conclusions have been confirmed by the recovery of added phosphate in trichloroacetic acid filtrates containing excess phosphotungstic acid.

Occasionally, we have been fortunate enough to secure cell preparations which were so thoroughly freed of seminal fluid protein by washing that the addition of phosphotungstic acid was unnecessary. Chemical analyses under these conditions gave phosphate values identical with those in the presence of phosphotungstic acid.

Phosphate determinations were made by the method of Fiske and Subbarow (5). Lactic acid was determined by the method of Barker and Summerson (6). Glucose was determined by a combination of the Hagedorn-Jensen (7) and Folin-Malmros (8) methods, as follows: An aliquot of the trichloroacetic acid-phosphotungstic acid filtrate containing about

200 to 300 γ of glucose was treated with 1 per cent sodium carbonate to faint alkalinity, and then diluted to 10 cc. A 1 cc. portion of this was removed (the remaining 9 cc. were usually used for the lactic acid analysis) and placed in a Klett-Summerson colorimeter tube. 1 cc. of the Hagedorn-Jensen alkaline ferricyanide reagent was added, and the tube placed in a boiling water bath for 15 minutes. After cooling, 1 cc. of the Folin-Malmros ferric-iron-gum ghatti reagent was added, followed by water to a volume of 10 cc. The color was read in the photoelectric colorimeter after 10 minutes standing, with a green filter (540 m μ). From the reading of a suitable standard prepared at the same time, the glucose concentration was obtained.

TABLE I

Action of Human Spermatozoa on Adenosine Triphosphate (ATP)

Temperature, 38°; medium, Ringer-bicarbonate; volume, 1.2 cc.; cells washed once with Ringer's solution before use; pH 7.4.

Cell count $\times 10^4$	Time	Gas phase	Glucose	ATP P added	Inorganic P formed	Hydrolysis of ATP
	hrs.			γ	γ	per cent
167	4	N ₂ -CO ₂	+	95	95	100
167	4	O ₂ -CO ₂	+	95	98	103
152	4	N ₂ -CO ₂	+	99	95	96
152	4	O ₂ -CO ₂	+	99	97	98
205	3	N ₂ -CO ₂	+	82	77	94
205	3	"	-	82	84	102
215	4	"	+	99	95	96
107	3	"	-	95	91	96

Results

Adenosine Triphosphate—The phosphatase activity of human spermatozoa on adenosine triphosphate (ATP) is illustrated by the data of Table I. The adenosine triphosphate used in these experiments was prepared from rabbit muscle and characterized by the usual methods based on the ratio of nitrogen to phosphorus, and of labile phosphorus to total phosphorus. It was usually added at the concentrations indicated, in the form of a filtered or centrifuged solution of the calcium salt in Ringer's bicarbonate solution. Use of the sodium salt gave identical results.

In the first group of experiments, cells washed once with Ringer's solution were used. It was found that complete splitting of all the phosphorus from ATP invariably occurred. It appeared probable that contamination of the cells by adhering seminal fluid might account for these results, since seminal fluid is known to have a powerful and heterogeneous phos-

phatase activity (9). Accordingly, the experiments were repeated, with cells which had been washed three times with Ringer's solution, to remove as much of the adhering seminal fluid as possible.

The well washed cells proved to have a phosphate-splitting power corresponding to the liberation of between 1 and 2 of the 3 phosphorus atoms present in ATP (Table II). The variation here appeared likely, due to incompleteness of reaction, since the greatest number of cells with the smallest concentration of substrate gave the highest per cent of splitting. To test this, several experiments were run with a minimal amount of added ATP, and the rate of phosphate hydrolysis followed. The results of these experiments are indicated by Fig. 1, which shows clearly that under optimal conditions for progression of the reaction to completion the cells split two-thirds of the total phosphorus from ATP, and no more.

TABLE II

Action of Human Spermatozoa on Adenosine Triphosphate (ATP)

Temperature, 38°; medium, Ringer-bicarbonate; pH 7.4; volume, 1.2 cc., cells washed three times with Ringer's solution before use.

Cell count × 10 ⁶	Time	Gas phase	Glucose	ATP P added	Inorganic P formed	Hydrolysis of ATP
	<i>hrs.</i>			<i>γ</i>	<i>γ</i>	<i>per cent</i>
64	3	N ₂ -CO ₂	+	182	50	28
182	3	"	+	82	37	45
108	3	"	+	89	40	45
108	3	O ₂ -CO ₂	+	89	39	44
92	4	N ₂ -CO ₂	—	115	47	41
120	3	"	—	55	37	67

This would correspond to the formation of adenylic acid from adenosine triphosphate. Although we have not isolated adenylic acid from the reaction mixture, all our evidence indicates that this is the fundamental reaction that occurs when human spermatozoa which have been washed free from contaminating seminal fluid are incubated with ATP. The significance of this reaction will be discussed below.

The action of human spermatozoa on various other phosphate esters of possible significance in intermediary carbohydrate metabolism has also been studied. Representative results are summarized in Table III. The hexose-6-phosphate ("Embden ester"), together with a sample of glucose-1-phosphate, was kindly supplied by Dr. Carl Cori. Not included in Table III are results on fructose-1,6-diphosphate and on sodium β -glycerophosphate, because they were essentially identical with those shown.

In every case, it was found that, while the once washed cells produced

considerable hydrolysis of all the phosphate esters studied, when the cells were washed three times their ability to split phosphate from these com-

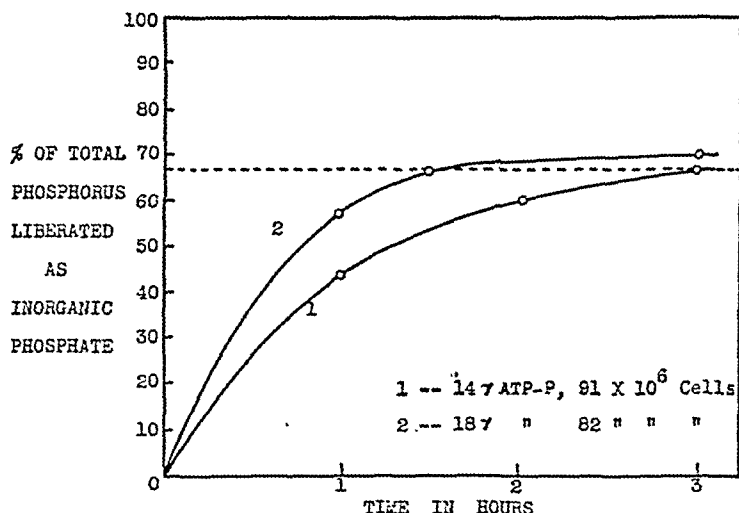


FIG. 1. Time relationship of action of spermatozoa on adenosine triphosphate. Temperature, 38°. Ringer-bicarbonate medium, anaerobic, pH 7.4; volume, 1.15 cc.; cells washed three times.

TABLE III

Action of Human Spermatozoa on Hexose-6-phosphate and Glucose-1-phosphate
Temperature, 38°; medium, Ringer-bicarbonate; pH 7.4; volume, 1.2 cc.; gas phase, N₂-CO₂; no glucose in medium.

	Cell count X 10 ⁴	Cells washed	Time	Ester P added	Inorganic P formed	Hydrolysis of ester
		times	hrs.	γ	γ	per cent
Hexose-6-phosphate	215	1	4	112	83	74
	107	1	4	112	34	30
	100	3	3	256	4	1.5
	58	3	3	167	3	1.3
	174	3	3	220	5	2.2
Glucose-1-phosphate	157	3	3	112	2	1.7
	92	3	4	103	3	2.9
	120	3	3	72	2	2.8

pounds was completely lost. Whether this is due to inability of the substrate to penetrate the cell structure or to some other reason is not known.

It is clear, however, that of the various phosphate esters studied only adenosine triphosphate¹ is acted upon by the cells.

DISCUSSION

The presence of ATP or of other phosphate esters studied does not have any effect either in depressing or elevating the motile activity of the spermatozoa. As has been shown (2), the motility of human spermatozoa fails rapidly in the absence of glucose. This failure of motility is not arrested in the presence of ATP dephosphorylation, so that if any energy is released in this reaction it is not available for motile activity. It should also be emphasized that none of the glucose esters studied maintained motility in the absence of free glucose, or if they were not dephosphorylated by adhering seminal fluid. For example, the Cori ester did not sustain the motility of thrice washed cells but proved an excellent substrate with once washed cells. Lastly, the presence of ATP or the other esters did not have any effect in raising or lowering lactate production in the presence of glucose. The reason for this apparent specific enzymatic activity of human spermatozoa on ATP is, therefore, not obvious at this time.

It was considered possible that, in the presence of suitable acceptors, the phosphate split from ATP by the spermatozoa adenosinetriphosphatase would be transferred. This did not prove to be the case. A variety of possible phosphate acceptors including glucose, creatine, and hexose monophosphate was added to the incubation medium in the presence of ATP, but in each instance, the inorganic phosphate recovered was the same as in the absence of the acceptors; nor was there any manometric evidence of phosphate transfer. These data do not agree with the recent findings of Mann (10) with ram spermatozoa.

The adenosinetriphosphatase activity of the seminal fluid, through which all of the phosphate bonds of ATP are hydrolyzed, is not associated with the "acid phosphatase" activity of the seminal fluid described by Gutman and Gutman (9). We have determined the activity of a partially purified preparation of this enzyme² in the presence of ATP and found it to be inactive. However, it splits the phosphate of β -glycerophosphate and the hexose phosphates very rapidly and completely. It is probable that the adenosinetriphosphatase activity of the seminal fluid is mediated through a phosphatase hitherto undescribed for this fluid.

¹Since the completion of these studies, we have been able to test the action of thrice washed human spermatozoa on acetyl phosphate which was kindly supplied to us by Dr. F. Lipmann. The washed spermatozoa proved capable of rapidly hydrolyzing this compound. Further studies on this substrate are in progress.

²We are indebted to Dr. Gutman for a generous sample of the partially purified phosphatase.

SUMMARY

1. Human spermatozoa washed free of adhering seminal fluid are capable of hydrolyzing adenosine triphosphate to produce adenylic acid. Various other phosphate esters of recognized significance in carbohydrate metabolism are not hydrolyzed under the same conditions.

2. The hydrolysis of adenosine triphosphate does not modify the normal production of lactic acid from glucose by the cells.

3. The liberation of phosphorus from adenosine triphosphate is not accompanied by phosphate transfer; nor is any energy yielded by this reaction made available for the maintenance of motility.

4. Human seminal fluid contains an enzyme or enzymes catalyzing the splitting of phosphate from all the phosphate esters studied.

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PHOSPHOPROTEIN PHOSPHATASE, A NEW ENZYME FROM THE FROG EGG

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In the nutrition of the young, the rôle of phosphoproteins is, without doubt, an important one. As casein in milk, or as the yellow yolk of many vertebrate and invertebrate eggs, phosphoprotein occurs in substantial quantities. In the frog egg, for example, the yolk platelets occupy almost half the volume of the cell. According to McClendon (5), the yolk of the frog egg consists of phosphoprotein bound to lipide; thus the platelets are a ready source of protein, lipide, and phosphorus. And yet, in spite of the obvious importance of phosphoprotein, little information is available about the manner of its utilization by the developing embryo. It has been assumed that ordinary proteolytic enzymes degrade the protein, and that ordinary phosphomonoesterases split the phosphate from phosphopeptone or from phosphoserine.

In the course of an investigation of the distribution of enzymes in the frog egg, a new enzyme (or enzymes) was discovered which is apparently capable of splitting inorganic phosphate from the intact protein molecule. It is the purpose of this paper to present the experiments which led to this conclusion.

EXPERIMENTAL

Methods—Ovarian eggs of the common leopard-frog, *Rana pipiens*, or, in a few cases, those of the bullfrog, *Rana catesbiana*, were used. The ovaries were removed, soaked for half an hour or more in 0.1 M sodium citrate, blotted rapidly on filter paper, and weighed to the nearest cg. They were then suspended in fresh citrate solution¹ and ground with a homogenizer patterned after that of Potter and Elvehjem (6) but modified in that the

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¹For the general purpose of the investigation sodium citrate appeared to be the best suspension medium for the homogenate, since the cytoplasmic granules of the egg protoplasm appeared microscopically to be in good condition, and these granules could be isolated in an essentially quantitative manner by differential centrifuging. Brei prepared in sodium chloride or potassium chloride was apparently not homogeneous and could not be fractionated by differential centrifuging in a satisfactory manner.

rotating pestle was smooth and fitted loosely in the surrounding test-tube. This modification was found to be particularly suitable for homogenizing frog ovary, since the large, fully developed eggs are readily ruptured, while the connective tissue sheet and the small cells embedded in it remain intact. This sheet becomes wrapped about the stem of the pestle, is later removed, and its weight subtracted from that of the whole ovary.

In most of the experiments reported here, the homogenate was incubated in citrate buffer for a suitable time and then treated with trichloroacetic acid to precipitate proteins. Unfortunately, certain substances of the frog

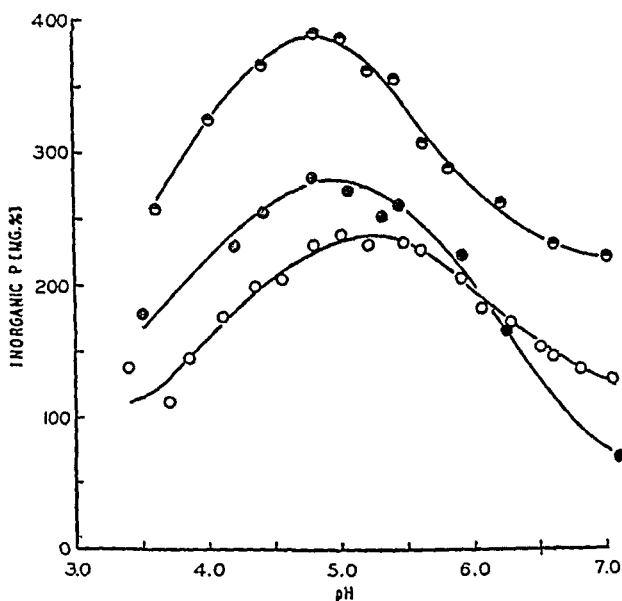


FIG. 1. Inorganic phosphate in autolyzed ovarian homogenate. Half circles, *Rana pipiens*, 60 minutes, 37°; solid circles, *Rana pipiens*, 5 minutes, 23°; open circles, *Rana catesbiana*, 60 minutes, 37°.

egg are not precipitated readily, and to obtain a water-clear filtrate it was often necessary to allow precipitation to continue overnight before filtering or centrifuging. Undesirable as this procedure may be, no way of avoiding it was found.

Phosphate was determined according to the method of Fiske and Subbarow (2). Although citrate may, in sufficient concentration, delay the development of color in this method, no difficulties were experienced in the experiments reported here. Phosphate is expressed as mg. of phosphorus per 100 gm. of wet weight.

General Characteristics of Reaction—In an attempt to demonstrate in

the frog egg homogenate a phosphomonoesterase hydrolyzing sodium β -glycerophosphate, extremely high values of inorganic phosphate were consistently found in the brei incubated without added substrate as a control. As much as 500 mg. per cent of inorganic phosphate were found after an incubation period of 1 hour. Moreover, there was a close dependence upon pH, maximum phosphate being found in the neighborhood of pH 5.0. Fig. 1 illustrates typical data obtained in three independent experiments. The high value of phosphorus shown in the uppermost

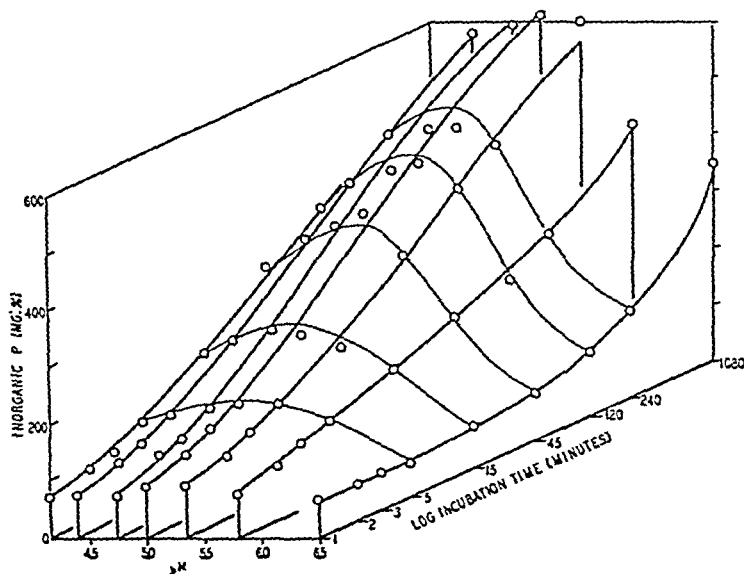


FIG. 2. Liberation of inorganic phosphate in autolyzing ovarian homogenate. A logarithmic time scale is used for convenience; the curves are empirical.

curve at pH 7.0 may be erroneous, since the reaction is very sensitive to pH in this region and pH was calculated rather than determined in this experiment. There is virtually no hydrolysis in alkaline solutions. Since all of these experiments were carried out on the crude autolyzing brei, considerable variability is to be expected, but the general characteristics are reproducible.

Fig. 2 illustrates a more complete experiment, in which the course of the reaction was followed for a considerable period of time at the different pH values indicated. For convenience a logarithmic time scale is used, since after the first 15 to 20 minutes the reaction proceeds very slowly.

These data strongly suggest that some naturally occurring phosphorus

compound, presumably an ester, is hydrolyzed. That this is an enzymatic hydrolysis rather than a simple acid hydrolysis is indicated by the dependence upon pH, the fact that the reaction is stopped by the addition of trichloroacetic acid, and by the great speed of the reaction. Indeed, as Fig. 1 demonstrates, as much as 250 mg. per cent of inorganic phosphorus can be liberated in 5 minutes at room temperature. It has also been shown that the reaction can be prevented by heating the brei.

Nature of Substrate—Assuming the reaction to be an enzymatic hydrolysis of a naturally occurring phosphate ester, attempts were made to identify the substrate. No significant changes could be demonstrated in the acid-soluble esters hydrolyzable by 1 N hydrochloric acid. A general fractionation of the brei was therefore made as follows. Aliquots of the autolyzing brei were pipetted into trichloroacetic acid at suitable time intervals. Inorganic phosphorus was determined and, after ashing with perchloric acid, total acid-soluble phosphorus was determined. The difference between these values was taken to be organic acid-soluble phosphorus. Since the trichloroacetic acid filtrate was perfectly clear, it was assumed that all the lipide was carried down with the precipitated protein.² The precipitate was extracted two or three times with hot alcohol-ether, and the residue considered to be protein. After all traces of alcohol and ether were driven off on a steam bath, the lipide and protein fractions were ashed with perchloric acid and phosphorus was determined.

A representative experiment carried out at pH 5.0 is shown in Fig. 3. The obvious rise in inorganic phosphorus is completely accounted for by the fall in protein phosphorus, while there is no change in the lipide fraction, and but a slight increase in the organic acid-soluble phosphorus. This increase has not been extensively investigated. There seems little reason to doubt that protein phosphorus is the source of the inorganic phosphate released in the autolyzing brei. It will be seen from Fig. 3 that part of the protein phosphorus is resistant to hydrolysis. The interpretation of this will be discussed below.

There are in the frog egg two proteins which contain substantial amounts of phosphorus, nucleoprotein, and phosphoprotein. Either might be the substrate for this enzyme. To distinguish between these two, whole brei was incubated alone, with added casein, or with added ribose nucleic acid. Fig. 4 shows the phosphorus liberated in 30 minutes from the added substrates as well as from the natural substrate present in the autolyzing brei. A full interpretation of these data cannot be given at present, but

*This assumption is doubtless correct, since a good deal of lipide is bound to protein and thus would be precipitated directly. The free fat droplets appear to be covered by a film of protein, and might be precipitated directly, but would in any case be carried down mechanically with the bulky protein precipitate.

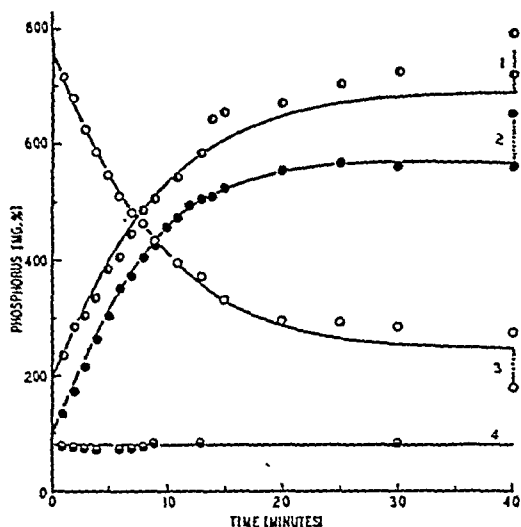


FIG. 3. Change in distribution of phosphorus in homogenate incubated at 23°, pH 5.0. Curve 1, total acid-soluble phosphorus; Curve 2, inorganic phosphorus; Curve 3, protein phosphorus; Curve 4, lipid phosphorus. The points at the end of the dotted lines are the values at 24 hours. The curves are empirical.

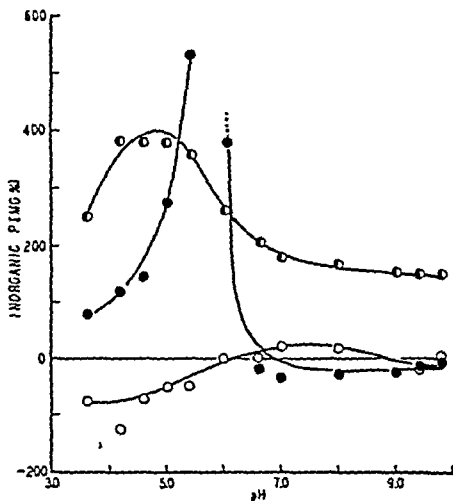


FIG. 4. Effect of the enzyme on various substrates. Half circles, natural substrate; solid circles, casein; open circles, yeast nucleic acid. Incubation time, 30 minutes; the curves are empirical.

there are several obvious features. The autolysis of the naturally occurring substrate is well illustrated but the high level of inorganic phosphate obtained from pH 7.0 to 10.0 is probably an artifact due to the rapid hydrolysis occurring during homogenization. Obviously there is a tremendous increase in inorganic phosphate when casein is added as a substrate, and the highest point on the curve may not be shown, since the reaction was not carried out at close enough intervals near the pH optimum. The general form of the casein curve seems essentially that of the autolytic curve, although the optimum is somewhat shifted, possibly due to the relative insolubility of casein at pH 5.0. Clearly, casein can serve as a substrate for this enzyme, which may therefore be called a phosphoprotein phosphatase. This observation has been repeatedly confirmed. Casein has been used routinely as a substrate with absolutely consistent results. The natural substrate in the egg is therefore phosphoprotein and this is present in large amounts in the yolk platelets.

Nucleic acid is evidently not hydrolyzed; as a matter of fact, less phosphate seems to be liberated from the yolk when nucleic acid is present. Presumably, the unhydrolyzable protein phosphate noted in Fig. 3 is largely nucleoprotein phosphate.

Localization of Enzyme—Since the work thus far reported was part of a general study of the distribution of enzymes within the frog egg, a few preliminary experiments were carried out to see whether the enzyme was bound to the yolk platelets, obviously the natural substrate. A typical experiment will illustrate the results obtained. Whole brei was centrifuged and the yolk-free brei was decanted from the sedimented yolk. The yolk was then washed three times by centrifugation in 10 volumes of fresh sodium citrate, so that any enzyme not bound to the yolk particles would be washed away. Finally the yolk was resuspended in citrate solution. Samples of the yolk suspension and of the yolk-free brei were set aside and the balance of each heated to 80° for 30 minutes to inactivate the enzyme. Aliquots of the heated and unheated yolk and yolk-free brei were then incubated at 37° for 30 minutes at pH 5.0 in the presence of the following substrates: none, heated yolk suspension, vitellin prepared from hen's eggs, according to the method of Calvery and White (1), sodium β -glycerophosphate, and disodium phenyl phosphate. Table I shows the amount of phosphate liberated from the added substrate by the action of the enzyme. These figures were obtained by subtracting from the total inorganic phosphate found in the digest the inorganic phosphate found in the substrate solution after incubation, and the inorganic phosphate liberated by autolysis in the enzyme solution. The latter figure is given in the first column of Table I.

A number of conclusions are clearly indicated by these data. (1) The

activity of the enzyme is destroyed by heating. The deviations from zero when heated enzyme solutions were used are not significant. (2) The enzyme is bound in part to the yolk platelets. The alternative explanation, that the enzyme is adsorbed during the course of preparation, is not very probable. The dilution of the egg protoplasm which takes place during preparation of the brei and the washing of the yolk are conditions which would tend to remove the enzyme rather than cause it to be adsorbed. In the absence of kinetic data, it is not possible to determine how much enzyme remains adsorbed to the yolk particles. Furthermore, the amount eluted during the course of preparation is indeterminate, so that a qualitative demonstration of the physical association of the enzyme and substrate is all that is possible at the present time. (3) The enzyme attacks all three phosphoproteins tested with considerable vigor, but has relatively little activity on either glycerophosphate or phenyl phosphate.

TABLE I
Localization of Enzyme and Effect on Various Substrates

Enzyme source	P liberated (mg. per 100 gm.) in 30 minutes, 37°, pH 5.0					
	Substrate					
	None	Yolk (heated)	Casein	Vitellin	Glycerophosphate	Phenyl phosphate
Yolk-free brei	45	83	359	108	0	14
Same (heated)	5	1	23	-11	0	-8
Yolk	86	94	219	99	3	-11
" (heated)	0	-5	29	-16	-7	-21

Kinetics—A number of experiments have provided data which are suitable for a preliminary analysis of the kinetics of the autolytic reaction. Chemically, one would expect a pseudomonomolecular reaction, since the reaction catalyzed is presumably a simple hydrolysis of serine phosphate, which according to Lipmann (4) is the only phosphorus compound present in casein. One may follow the disappearance of the substrate ($A - x$) directly, by analysis of protein phosphorus, with suitable correction for unhydrolyzable phosphorus, or indirectly through the appearance of inorganic phosphate (x). To the extent that organic acid-soluble phosphorus is formed from phosphoprotein, the results of these two methods will differ. However, the general results obtained by either approach are the same, as Fig. 5 demonstrates.

This figure is a plot of $\log A - x$ against time for four independent experiments. The solid circles represent data obtained by a direct analysis of protein phosphorus, while all the other experiments are based on the

appearance of inorganic phosphate. The reaction appears to follow monomolecular kinetics quite exactly for 15 to 20 minutes, but there is apparently a sudden break in the rate of the reaction which then proceeds more slowly than would be predicted by theory. Sufficient data about the later stages of the reaction are not available to justify any particular theoretical interpretation. Hence the straight line following the break should be regarded as empirical. However, considering the complicated physical conditions which prevail in the crude brei, with much of the substrate solid and part of the enzyme bound, it is surprising, indeed, that the reaction has predictable kinetics at all.

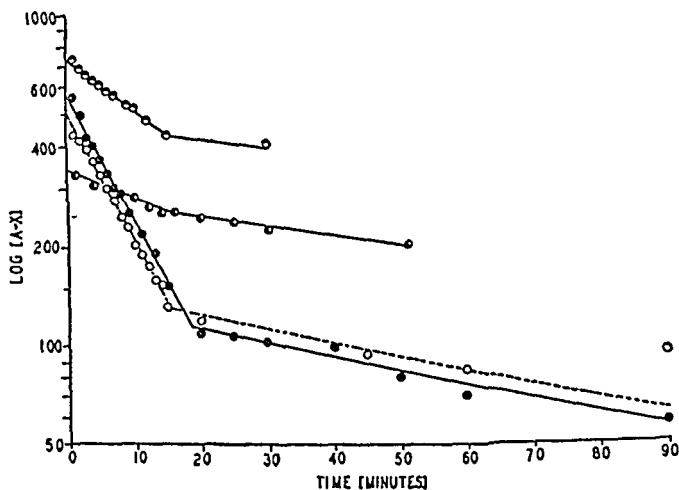


FIG. 5. Kinetics of the autolytic reaction. The solid circles are based on the direct analysis of protein phosphorus; other data on the appearance of inorganic phosphorus. The initial straight line is based on the equation; $kt = \log(A/(A-x))$; the straight line following the break is empirical.

These kinetic results lend support to the idea, suggested by the speed of the reaction, that the hydrolysis of phosphorus from phosphoprotein is a direct one without the intervention of preliminary proteolytic activity. Direct support for this idea is provided by the fact that no increase in acid-soluble nitrogen has been detected.

DISCUSSION

The above data seem to justify the conclusion that the ovarian eggs of the leopard-frog contain an active enzyme which attacks phosphoprotein with the liberation of inorganic phosphate. The eggs of the bullfrog likewise contain this enzyme, but in a single experiment it was not demonstrated in the hen's egg. It is to be expected that the enzyme may occur

in other eggs of vertebrates and invertebrates which contain large amounts of phosphoprotein.

Detailed studies of the specificity of this enzyme are still needed, but the phosphatases described in the literature do not show the properties displayed by the frog egg enzyme. Most authors report that phosphatases have no action on casein. Kay (3) and Rimington and Kay (8) state that bone and kidney phosphatases do not split phosphate from casein, although the phosphopeptone resulting from tryptic hydrolysis is attacked by both enzymes. Taka-phosphoesterase causes weak hydrolysis of phosphopeptone at pH 5.6, according to Sadanitzu (9) and Sorimati (11). Travia and Veronese (12) report that an alkaline phosphatase from ox spleen does attack casein. Intestinal phosphatase has no action on casein, according to Schmidt and Thannhauser (10). Thus it would seem that most phosphatases have little or no action on casein. Certainly no phosphatase previously described attacks phosphoprotein more readily than glycerophosphate.

No exact study of the kinetics of this reaction or of the influence of cytological structure upon it can be made until the enzyme has been purified. It seems remarkable, however, that the enzyme is able to liberate phosphate with such speed when it is considered that two proteins are involved, and particularly when it is realized that much of the substrate is not in solution. The fact that the reaction appears to proceed in two stages is reminiscent of the observations of Rimington and Kay (8) and Rimington (7), who noted that only part of the phosphate could be split from phosphopeptone by bone phosphatase or alkali, whereas all could be released by kidney phosphatase.

This enzyme may prove to be a useful tool in studying the chemical and physical properties of phosphoproteins. Thus, information as to the mode of linkage of phosphorus in casein might be obtained. Studies on dephosphorized proteins might be facilitated by the specific dephosphorylation method in place of the alkali treatment heretofore used.

The autolytic reaction which occurs so dramatically when the frog egg is ruptured must somehow be held in check in the intact cell, for the eggs remain in a relatively static state throughout the entire winter. After fertilization of the egg, small quantities of phosphate must presumably be liberated gradually or from time to time to meet the metabolic needs of the embryo. The mechanism by which the enzyme is held in check or controlled is uncertain. A favorite explanation in such cases is that enzyme and substrate are not in contact. This seems not to be true here, for a considerable proportion of the enzyme appears to be bound directly to the yolk particles. Other possible interpretations include inhibitors within the cell, addition of activators on homogenization, or other altera-

tions in the physical and chemical conditions. At present, it would seem that the pH of the cell may be adverse, since near neutrality the activity of the enzyme is profoundly affected by small changes in pH. On fertilization perhaps enough acidity develops to activate the enzyme.

I am grateful for helpful discussions with the late Dr. Carl L. A. Schmidt and Dr. D. M. Greenberg, and for the cooperation of Miss Mary Cregar who assisted me in some of these experiments.

SUMMARY

1. A rapid, heat-labile autolytic reaction has been demonstrated in homogenates of ovarian frog eggs, resulting in the liberation of large amounts of inorganic phosphate.

2. This reaction occurs over a broad range in acid solutions with an optimum near pH 5.0.

3. There is a decrease in protein phosphorus which completely accounts for the increase in inorganic phosphate, while organic acid-soluble phosphorus and lipid phosphorus remain essentially unchanged.

4. Phosphate is split readily from added casein, vitellin of hen's eggs, and yolk of frog eggs, while nucleic acid, glycerophosphate, and phenyl phosphate are attacked slowly, if at all.

5. It is concluded that the reaction is enzymatic and that the enzyme is best characterized as a phosphoprotein phosphatase. A survey of the literature indicates that an enzyme with this specificity has not previously been described.

6. Preliminary data on the kinetics of the reaction are presented.

7. Part of the enzyme seems to be bound directly to the yolk platelets which contain the natural substrate.

8. The rôle of the enzyme in metabolism is briefly discussed.

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STUDIES ON CHOLINE ACETYLASE

III. ON THE PREPARATION OF THE COENZYME AND ITS EFFECT ON THE ENZYME*

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In 1943, an enzyme, choline acetylase, which forms acetylcholine in the presence of adenosine triphosphate was extracted from brain (Nachmansohn and Machado (1)). The enzyme may be prepared either from fresh tissue or from acetone-dried powder (Nachmansohn and John (2, 3)). If prepared in the latter way, choline acetylase is practically completely separated from cholinesterase, which becomes inactive by treatment with acetone (4). Shortly after the discovery of choline acetylase, it was found that this enzyme rapidly becomes inactive on dialysis (Nachmansohn, John, and Waelsch (5)). *l* (+)-Glutamic acid and citric acid in 0.02 *M* concentration may reactivate it partly. Stronger but not yet complete reactivation was obtained with cysteine. The experiments suggest that the enzyme requires a coenzyme for its activity.

The coenzyme has now been extracted and partly purified. In this paper the preparation of the coenzyme, its effects, and those of other compounds on the enzyme will be described.

Methods

The methods used for the preparation of the enzyme were essentially the same as described in previous communications (1, 3). Rabbit brains were used in preparing the acetone-dried powder which was kept in a deep freezer at -20° and remained fairly constant for many weeks. In view of its high activity, the enzyme used per vessel was equivalent to an extract of 70 to 100 mg. of powder.

Choline, acetate, adenosine triphosphate, eserine, potassium, and cysteine were added in the same concentration as previously (3). Fluoride was added only to extracts prepared from fresh tissue. The total volume of the enzyme solution during incubation was kept between 4.5 and 5.0 cc. The time of incubation was generally 20 minutes; in a few cases, *e.g.* the optic nerve, it was 30 minutes. At the end of the incubation period, the enzyme

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solution was inactivated in the usual way and the amount of acetylcholine formed was tested by bioassay on frog rectus abdominis. Because of the high amounts of acetylcholine formed, the solution was generally diluted 50 to 100 times or more, so that interference from other compounds can be excluded.

Results

Preparation of Coenzyme—In contrast to the enzyme, which occurs only in nerve tissue, the coenzyme has been found in various organs. It is present in brain, liver, heart, and skeletal muscle.

An active preparation of the coenzyme was obtained in the following way. The tissue was ground in a Waring blender in a volume of water

TABLE I

Reactivation of Dialyzed Choline Acetylase by Coenzyme Prepared from Different Tissues

The choline acetylase was always extracted from the powder of acetone-dried rabbit brains. No coenzyme was added to the undialyzed enzyme.

	Experiment 1, rabbit brain	Experiment 2, rabbit brain	Experiment 3, rabbit heart	Experiment 4, pigeon breast muscle	Experiment 5, pig heart
	Acetylcholine formed per gm. per hr.				
	mg.	mg.	mg.	mg.	mg.
Undialyzed.....	0.70	0.70	0.70	0.47	1.29
Dialyzed + K + cysteine.....	0.45	0.55	0.40	0.26	0.45
" + coenzyme.....	0.93	1.20	1.00	1.06	1.31

equivalent to 1 to 2 times its weight. The suspension was heated to 100° for a few minutes and filtered. Acetic acid was added to remove proteins. After filtration and neutralization, an active solution was obtained (see Table I, Experiment 1). In a few cases this solution was poured into a 20-fold volume of ice-cooled acetone. The precipitate was filtered and dried and the powder was redissolved in buffer. The reactivation of the dialyzed enzyme by this solution is shown in Table I, Experiments 2 and 3.

A partial purification of the coenzyme was obtained by precipitating with barium hydroxide and redissolving with sodium sulfate. The addition of the barium precipitates all the activity, so that a highly active preparation of the coenzyme is obtained. The coenzyme used in Experiments 4 and 5 of Table I was purified in this way.

Since in most other experiments the coenzyme was obtained by a barium

precipitation of pig heart extracts, a typical preparation may be described in detail.

480 gm. of fresh pig hearts were homogenized in 930 cc. of water, boiled, passed through gauze, and filtered. 960 cc. of solution were obtained. 96 cc. of 3 per cent acetic acid were added. The precipitate obtained was filtered and the clear solution neutralized with 96 cc. of 0.5 N NaOH. 200 cc. of this solution were precipitated with 25 cc. of 0.1 N $\text{Ba}(\text{OH})_2$. The precipitate was centrifuged, the supernatant discarded, and the precipitate dissolved in 25 cc. of 0.1 N Na_2SO_4 .

The coenzyme could be obtained from this solution in powder form as the sodium salt by dehydration in a vacuum in dry ice. About 2 to 3 mg. of the

TABLE II

Effect of Acid and Alkali on Coenzyme of Choline Acetylase

The coenzyme was treated either with NaOH or with HCl, the final concentration being 0.1 N. At the end of the period indicated the solution was neutralized and the coenzyme added to the enzyme solution.

Treatment of coenzyme	Duration	Temperature	Acetylcholine formed	
	min	°C	mg per gm. per hr.	mg per gm. per hr.
Control			1.02	1.00
" + coenzyme			1.69	1.56
Boiled	60	100		1.40
HCl	30	100	1.23	1.27
NaOH	30	100	1.05	1.05
HCl	60	23	1.70	
NaOH	60	23	1.29	

powder per cc. were required to obtain the maximal effect; however, a great part of this salt is undoubtedly sodium sulfate.

The stability of the purified coenzyme in acid and alkali was tested in the following way: HCl or NaOH was added to the solution containing the coenzyme, so as to make the final concentration 0.1 N. The solution was kept for 30 minutes in a sealed test-tube in a boiling water bath or for 60 minutes at room temperature (23°). The results of the experiments are summarized in Table II. Boiling for 60 minutes decreased the activity of the coenzyme about 30 per cent. After treatment with 0.1 N HCl at 100°, the loss of activity was 66 per cent in one experiment, 52 per cent in the second. With 0.1 N NaOH nearly the total activity of the coenzyme was lost in 30 minutes at 100°. At room temperature 0.1 N HCl had no effect, whereas 0.1 N NaOH destroyed more than half of the activity.

Effect of Coenzyme on Dialyzed Enzyme—If the enzyme is dialyzed for 2 hours, it becomes practically inactive. No measurable formation of

acetylcholine is observed on the addition of eserine, choline, acetate, and adenosine triphosphate. If potassium and cysteine are added, about 60 to 70 per cent of the initial activity may be restored, as can be seen from the figures in Table I. If in addition to the other compounds mentioned the coenzyme is added to the dialyzed solution, the rate of formation is even higher than in the undialyzed extracts to which no coenzyme was added.

However, as may be seen from the later observations, the activity of the undialyzed enzyme would have been markedly higher if the coenzyme had been added. In Experiment 5, in which the dialysis was continued for 5 hours, only 35 per cent of the initial activity was restored with potassium

TABLE III

Effect of Coenzyme on Undialyzed Choline Acetylase Extracted Either from Fresh Tissue or from Acetone-Dried Powder

Species	Tissue	Kind of extract	Acetylcholine formed	
			Without coenzyme	With coenzyme
			γ per gm. per hr.	γ per gm. per hr.
Guinea pig	Brain	Fresh	152.0	210.0
Rabbit	"	Acetone-dried powder	117.0	160.0
"	Sciatic nerve	Fresh	685.0	1330.0
"	Optic nerve	"	88.0	110.0
			16.0	21.0
Lobster	Abdominal chain	"	13.9	44.5
			18.8	92.7
<i>Electrophorus electricus</i>	Electric tissue	Acetone-dried powder		160.0
			124.0	222.0

and cysteine alone, whereas in the presence of coenzyme the synthesis was as strong as before dialysis.¹

Effect of Coenzyme on Undialyzed Enzyme—The dilution of coenzymes in extracts is frequently the limiting factor of the rate of enzyme activity; so that it is important to test the rate of acetylcholine formation by choline acetylase in the presence of the coenzyme. Moreover, for reasons discussed later, it is of physiological interest to obtain some information about the potential rate of acetylcholine formation in certain nerve tissue. Table III gives a summary of some of the data on the activity of undialyzed

¹ When these data were presented at the symposium of the New York Academy of Sciences on February 8 and 9, 1946 (6), F. Lipmann reported that he too had found the coenzyme simultaneously and independently (7). He has obtained a higher degree of purification. His observations seem to indicate that the coenzyme is a nucleotide.

choline acetylase in the presence of the coenzyme. In all cases tested, the addition of the coenzyme markedly increased the rate of acetylcholine formation.

In solutions prepared from freshly homogenized guinea pig brain, the activity increased 30 to 40 per cent in the presence of the coenzyme. In extracts from the powder of acetone-dried rabbit brain, the increase varied between 50 and 100 per cent. Table III shows only one figure, but additional data may be found in Table V.

In previous communications (8, 9), it was reported that choline acetylase is present in rather high concentrations not only in brain but also in peripheral fibers free of synapses and cell bodies. Rabbit sciatic nerve may form 70 to 90 γ of acetylcholine per gm. of fresh tissue per hour. In the presence of the coenzyme, the rate of formation in extracts from rabbit sciatic nerve is still further increased. In view of the high amount of fat and connective tissue in this nerve, the rate appears surprisingly high and consistent with the concept, supported by a large body of evidence, that acetylcholine has an essential rôle in the propagation of the nerve impulse.

The presence of choline acetylase in the optic nerve has also been tested. Complete absence of acetylcholine had been reported in this nerve and in the dorsal roots (10). Cholinesterase is present in both afferent and efferent nerves in concentrations of the same order of magnitude (11, 12). Acetylcholine is an extremely labile compound in living cells, whereas cholinesterase is a stable enzyme. Although choline acetylase is not as stable as is cholinesterase, it remains active for 15 to 30 minutes in the test-tube. It was therefore hoped that its presence in sensory nerves could be more easily demonstrated than the presence of the ester itself. One obstacle is the small amount of tissue available. 10 to 20 mg. of tissue (fresh weight) are sufficient for the determination of cholinesterase, but much larger amounts are required for testing choline acetylase activity. In the case of brain, about 400 to 500 mg. of tissue (fresh weight) appear to yield optimal values under the experimental conditions used. In order to obtain the necessary amounts of optic nerve for two vessels, the nerves of fifteen to twenty rabbits have to be obtained. On the basis of previous experience the possibility of deterioration of the enzyme during the 2 or more hours required for the operation cannot be excluded. In an experiment with smaller amounts of tissue, no formation was found, although this may not have been due exclusively to the small amount used. As may be seen in Table III, 1 gm. of optic nerve forms 15 to 20 γ of acetylcholine per hour. As with the rabbit sciatic nerve, the yield may increase with more experience. However, the fundamental question as to whether or not choline acetylase is present in sensory nerves has been decided in the affirmative.

A strong increase in activity has also been obtained by the addition of

the coenzyme to extracts prepared from the abdominal chain of the lobster. This is also the first evidence of choline acetylase in invertebrates.

Evidence for the presence of choline acetylase in electric tissue is complicated by the extraordinary high concentration of cholinesterase. Irregular results were obtained. Even in extracts prepared from acetone-dried powder of the electric organ of *Electrophorus electricus*, in which a great part of the cholinesterase activity is destroyed by the acetone, the activity of this enzyme is disturbing and interferes with the formation of acetylcholine in spite of the addition of eserine. It has been found that diisopropyl fluorophosphate (DFP), which is a strong inhibitor of cholinesterase, does not affect choline acetylase (Nachmansohn and John, unpublished experi-

TABLE IV

Effect of Mg and Mn on Undialyzed Choline Acetylase Extracted from Powder of Acetone-Dried Rabbit Brain

	Ion	Concentration	Acetylcholine formed	
			Experiment 1	Experiment 2
		M	<i>mg. per gm. per hr.</i>	<i>mg. per gm. per hr.</i>
Control			0.91	0.99
+ coenzyme			1.40	1.69
+ "	Mg	1×10^{-4}	1.53	
+ "	"	4×10^{-4}	2.15	1.99
+ "	"	8×10^{-4}		2.21
+ "	"	1.6×10^{-3}	1.70	2.06
+ "	"	3.2×10^{-3}	1.40	
+ "	Mn	1×10^{-4}		2.10
+ "	"	3×10^{-4}		2.15
+ "	"	9×10^{-4}		2.29

ments). In contrast to eserine, DFP may inhibit cholinesterase irreversibly, although this irreversible change requires a certain period of time when the reaction occurs at lower temperature (13). In experiments in the presence of 4 mg. of DFP per cc., the rate of formation of acetylcholine amounted to 120 γ per gm. of powder per hour. In this concentration and at the temperature and during the incubation period used, experimental tests showed that the cholinesterase was practically completely destroyed. In the presence of the coenzyme, the yield was nearly twice as high. In another experiment, the yield increased slightly less in the presence of the coenzyme. It appears probable, however, that both amounts are far below the optimal.

Effect of Mg and Mn—Since Mg and Mn frequently accelerate phosphorylations, the effect of these ions on the rate of acetylcholine formation

was tested. As may be seen from the figures in Table IV, Mg in concentrations of about 4 to 8×10^{-4} M definitely increases the rate of acetylcholine formation by the undialyzed enzyme. In higher concentrations, the effect becomes smaller. Mn, as known from many other observations, has the same effect.

The increase of choline acetylase activity in the presence of the coenzyme and Mg is also shown in the experiments summarized in Table V. The activity of the enzyme is approximately doubled if both substances are present.

TABLE V
Potentiating Effect of Presence of Both Coenzyme and Mg on Activity of Choline Acetylase

Powder No.	Control	Acetylcholine formed	
		+ coenzyme	+ coenzyme + Mg
		mg. per gm. per hr.	mg. per gm. per hr.
8		1.96	
	0.56	1.64	
	0.88	1.65	
	1.02	1.72	1.94
Average.....	0.82	1.74	1.94
11	0.91	1.40	2.15
		1.54	1.80
		1.49	
	0.80		1.63
	0.95		1.56
	0.95		1.90
Average	0.90	1.48	1.81
" of both series	0.87	1.63	1.83

If the enzyme is dialyzed, the effect of Mg appears to be about the same as that on the undialyzed enzyme (Table VI). The Mg cannot replace the potassium, as can be seen from the figures in Table VI.

Effect of High Speed Centrifugation on Choline Acetylase—Lipton has recently indicated that extracts of choline acetylase become clear and more purified by high speed centrifugation (14). Our experiments with high speed centrifugation confirm Lipton's observations that the solution becomes clear without losing its activity. There is also an increase in activity per gm. of protein (see Table VII).

One experiment was carried out in the following way. About 80 cc. of

enzyme solution, equivalent to 4 gm. of acetone-dried powder, were centrifuged for 30 minutes in a Svedberg ultracentrifuge at 20,000 R.P.M.

TABLE VI

Effect of Mg on Dialyzed Choline Acetylase

The period of dialysis was always 4 hours. The concentration of Mg was 0.0005 to 0.001 M.

Compounds added	Acetylcholine formed	
	Single values	Average
	<i>mg. per gm. per hr.</i>	<i>mg. per gm. per hr.</i>
Undialyzed control.....	1.86, 1.40, 1.96	1.74
Dialyzed + K + cysteine.....	0.62, 0.68	0.65
“ + “ + “ + coenzyme.....	1.20, 0.91, 1.16	1.09
“ + “ + “ + “ + “ +		
Mg.....	1.27, 1.29, 1.37	1.31
Dialyzed + cysteine + coenzyme + Mg.....	0.52, 0.59	0.56
“ + “ + Mg.....	0.16, 0.26, 0.53	0.36

TABLE VII

High Speed Centrifugation of Choline Acetylase

In the first series the enzyme activity was determined in the presence of the coenzyme, in the second in the presence of both coenzyme and Mg in 5×10^{-4} M concentration.

Time	R.P.M.	Portion	Protein	Acetylcholine formed	
				Per cc.	Per gm. protein
<i>min.</i>			<i>mg. per cc.</i>	<i>mg. per hr.</i>	<i>mg. per hr.</i>
35	20,000	Original solution	7.2	0.050	7.0
		Supernatant	5.9	0.045	7.7
		Pellets in 10 cc.	2.4	0	0
80	48,000	Top layer	2.0	0.017	8.5
		Middle layer	4.0	0.037	9.3
		Bottom “	9.2	0.075	7.6
		Pellets in 10 cc.	3.9	0.008	2.0
40 60	36,000 48,000	Original solution	10.9	0.0615	5.65
		Top layer		0.027	
		Middle layer	6.7	0.052	7.8
		Bottom “	10.9	0.113	10.4
		Pellets	16.3	0.006	0.4

The pellets were dissolved in 10 cc. of buffer and tested. There was practically no enzyme activity. The supernatant solution was then centri-

fuged for 80 minutes at 48,000 R.P.M. This centrifuged solution was divided into three equal portions (top, middle, and bottom layers) and analyzed. The pellets were analyzed separately. Most of the activity was found in the bottom layer. The acetylcholine formed per cc. per hour was about 4 to 5 times as high in the bottom as it was in the top layer. However, referred to the protein content, the activity did not vary considerably in the three layers. In another experiment the solution was centrifuged for 40 minutes at 36,000 R.P.M. and subsequently for 60 minutes at 48,000 R.P.M. and then treated in the same way as in the first experiment. As can be seen from the figures in Table VII, in this case a marked increase in activity per gm. of protein was obtained in the bottom layer. The

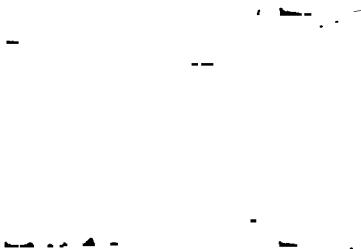


FIG. 1. Analytical test of choline acetylase in the ultracentrifuge. The bottom layer in the second experiment in Table VII was centrifuged for 158 minutes at 44,000 R.P.M. Exposures were made at 23, 34, 43, 55, 67, 81, 104, 121, 138, and 141 minutes after reaching full speed, but only four are reproduced: on the left side, the first and third; on the right, the fifth and seventh.

amount of acetylcholine formed per gm. rose from 5.65 to 10.4 mg., or 84 per cent. The distribution of the activity per cc. in the three layers was the same as in the first experiment.

An analytical test of the bottom layer of the second experiment showed only one component, as may be seen in Fig. 1. This of course cannot be taken as indicating the presence of a pure enzyme. The uncorrected sedimentation constant, S_r , of the protein was 40×10^{-13} , corresponding to a molecular weight of approximately 2,000,000. It probably is difficult to separate the enzyme from another protein, as has been the case with adenosinetriphosphatase and myosin. Still, recent experiments of Polis and Meyerhof (15) and of Price and Cori (16) have shown that such a separation may be possible. More experiments are necessary for the interpretation of our observation.

Inhibition by Naphthoquinones—In previous communications it was reported that α -keto acids are strong inhibitors of choline acetylase (2, 3). Another compound has now been found to inhibit the enzyme in relatively low concentrations; namely, methylnaphthoquinone, well known for its vitamin K activity. In a concentration of 0.005 M this vitamin decreases the enzyme activity about 40 per cent. Chargaff and Bendich have recently tested the effect of a few other naphthoquinones related to vitamin K on the coagulation of fibrinogen (17). We have tested whether some of these compounds have a more pronounced inhibitory effect on choline acetylase than does methylnaphthoquinone. Some typical results are given in Table VIII. By far the strongest action was observed with 2-

TABLE VIII
Inhibition of Choline Acetylase by Naphthoquinones

Compound	Concentration	Acetylcholine formed		Inhibition
		Control	Experiment	
	M	mg. per gm. per hr.	mg. per gm. per hr.	per cent
Methylnaphthoquinone	5×10^{-4}	1.86	1.11	40.0
1,2-Naphthoquinone-4-sulfonic acid (Na salt)	5×10^{-4}	1.86	1.33	28.5
1,4-Naphthoquinone-2-sulfonic acid (K salt)	2.5×10^{-4}	1.86	1.46	21.5
2-Methyl-1,4-naphthoquinone-8-sulfonic acid (K salt)	1.25×10^{-4}	1.63	0.64	60.6
		1.88	0.70	62.7
	6.25×10^{-5}	1.88	1.16	38.3
	3.13×10^{-5}	1.88	1.24	34.0
1,2-Naphthoquinone-4,8-disulfonic acid (K salt)	5×10^{-4}	1.56	1.15	26.3
Ninhydrin	5×10^{-4}	1.86	1.43	22.6

methyl-1,4-naphthoquinone-8-sulfonic acid, a compound, the synthesis of which was described recently by Bendich and Chargaff (18). The inhibition by the other compounds used did not differ markedly from that observed with vitamin K.

DISCUSSION

Choline acetylase requires in addition to the protein moiety, a coenzyme (according to F. Lipmann, possibly a nucleotide), adenosine triphosphate, and K and Mg (or Mn) ions for full activity. In the test-tube the system also requires cysteine. The enzyme has sulfhydryl groups which may be very easily oxidized, as was described by Nachmansohn and Machado

immediately after the discovery of the enzyme (1). The cysteine may be required to reduce the $-SH$ groups which were oxidized during the preparation. Whether or not the above compounds form the complete system has still to be ascertained. The restoration of the enzyme activity lost during dialysis was complete only in a few cases. It is of course possible that more cell constituents will be found which will further increase the enzyme activity.

It has been repeatedly stressed (19-21) that, in view of the high speed of the propagation of the nerve impulse and the small amounts of energy involved, great difficulties may be expected in any attempt to determine the cell constituents associated directly with this process. A new approach to the study of the chemical mechanism of nervous action was therefore initiated, based on investigations of the enzyme systems involved. During the years 1937 and 1938 it was shown that all nerve fibers, afferent or efferent, vertebrate or invertebrate, myelinated or non-myelinated, contain cholinesterase in a concentration of the same order of magnitude (11, 12). This appeared to be a support for the assumption that acetylcholine is metabolized in all fibers and has a function there. The observation of Loewi and Hellauer (10) that acetylcholine is present in efferent fibers but absent in sensory fibers has for many years been considered a major obstacle to the concept that acetylcholine plays an essential rôle in conduction (22). The evidence presented now that choline acetylase is present in the optic nerve is highly significant and a new additional support for the concept of an essential rôle of acetylcholine in conduction.

The rate of acetylcholine formation appears less important. 15 to 20 γ per hour are not of different order of magnitude from the 100 γ found in the sciatic nerve of the same species. In view of the experimental difficulties which were described above, it appears probable that with more experience and improved technique higher values will be obtained. In any case, the difference is not greater than that encountered for the concentration of cholinesterase in different nerves. In view of the complex nature of conduction, the release of acetylcholine can be only one link in a complex chain. Many other factors remain unknown. Conduction rates may differ 10 to 20 times in myelinated nerves of the same trunk (A and B fibers) (Grundfest (23)). It is not surprising to see 5-fold differences in the rate of an enzyme activity in different nerves. There is no apparent relationship between the rate of acetylcholine metabolism and the rate of conduction. So called non-myelinated fibers, although conducting at a lower rate, generally contain much more cholinesterase than do the myelinated fibers. Much more information is required before it will be possible to interpret these differences. The decisive fact is not the rate found but the evidence that choline acetylase is present in the optic nerve.

In several experiments in which the powder of acetone-dried rabbit brain was used, acetylcholine was formed at the rate of 2.0 to 2.2 mg. per gm. of powder per hour. Since 1 gm. of powder is equivalent to about 6 gm. of brain (fresh weight) about 350 γ of acetylcholine may be formed per gm. of brain per hour. Rabbit brain splits about 60 mg. of acetylcholine per gm. per hour (12). The difference between the rate of formation and removal is in this case, 150 to 200 times, whereas previously it was found to be 250 to 300 times, based on the data obtained with extracts of fresh guinea pig brains used under not quite optimal conditions (6). The discrepancy between the rates of formation and removal of acetylcholine has been discussed recently (6). If the release of acetylcholine is an essential event in the alterations of the nerve membrane during the passage of the impulse, the active ester has to be removed within a very brief period, within a millisecond or a fraction of a millisecond, possibly within 100 microseconds. No such speed has to be postulated for the resynthesis of the ester, which is an energy-requiring process and occurs during the recovery period. Considering all the factors involved (see the discussion mentioned above), the difference between the two rates appears to be well in the expected range.

SUMMARY

The preparation of the coenzyme of choline acetylase and some effects on the enzyme are described.

1. In contrast to the enzyme itself, present only in nerve tissue, the coenzyme appears to be present in a variety of tissues. It has been found in brain, liver, heart, and skeletal muscle.
2. The coenzyme can be purified by precipitating it as a barium salt.
3. After prolonged dialysis, the enzyme may be reactivated much more completely by the addition of the coenzyme than by K and cysteine alone.
4. The activity of the enzyme even when undialyzed and whether extracted from fresh tissue or from acetone-dried powder is considerably increased in the presence of the coenzyme.
5. Mg and Mn increase still further the activity of choline acetylase.
6. The presence of choline acetylase has been demonstrated in invertebrate nerves (abdominal chain of the lobster), in sensory nerves (optic nerve of the rabbit), and in electric tissue.
7. High speed centrifugation increases the degree of purity of the enzyme. The highest activity observed was 10.4 mg. of acetylcholine formed per gm. of protein per hour. This activity was found in the bottom layer after centrifugation at 48,000 R.P.M. The analytical test of this fraction showed only one component.

8. Methyl-naphthoquinone, the compound with vitamin K activity, inhibits choline acetylase. Of other naphthoquinones tested, 2-methyl-1,4-naphthoquinone-8-sulfonic acid was found to be a strong inhibitor.

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AN INVESTIGATION OF THE α - AND β -PHOSPHOLIPIDES*

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In a series of papers, Suzuki, Nishimoto, and Yokoyama (2-6) described the separation by solvents of cephalin lead salts and of lecithin cadmium salts from soy bean, human brain, and egg yolk into α and β fractions. They proved the identity of these fractions by hydrolyzing with barium hydroxide and then quantitatively determining the amount of β -glycerophosphate present by use of the relatively insoluble double salt discovered by Karrer and Salomon (7). Absence of this precipitate was taken as evidence of the presence of only the α isomer. These separations were carried out on highly purified preparations of cephalin and lecithin which represented only part of the original phospholipide.

This type of fractionation was later applied on a micro scale to crude cephalins and lecithins from tissues of dogs and rabbits by Yoshinaga (8), from organs of rats, cats, guinea pigs, and beef by Welch (9), and from the livers of fasting mice by MacLachlan *et al.* (10). No quantitative data were given by these authors to prove the identity of the glycerophosphate found in each fraction.

In this work phospholipides were prepared from egg yolk and calf brain, and separated into the α and β fractions, according to the methods in the literature. Extensive hydrolysis studies were carried out with both pure glycerophosphates and phospholipides, but these studies can be reported only very briefly here. The various cephalin and lecithin fractions were hydrolyzed and, after separation of the fatty acids, were analyzed by methods developed in this laboratory for α - and β -glycerophosphates (11) and nitrogenous constituents (12).

EXPERIMENTAL

Preparation of Material

Egg Yolk Phospholipide Fractions—Three sets of α and β fractionations of egg yolk phospholipides were carried out.

Egg Phospholipide I was prepared by following exactly the procedure described by Yokoyama (5). It was separated into Cephalin I and Lecithin

* The data in this paper are taken from a thesis submitted to the Graduate School of The University of Rochester in partial fulfillment of the requirements for the degree of Doctor of Philosophy. A preliminary report of these data has been given (1).

I by the methods of Nishimoto (6) and Yokoyama (5) and finally fractionated into α -Lecithin I and β -Lecithin I by the procedure of Suzuki and Yokoyama (3) and into α -Cephalin I and β -Cephalin I by the procedure of Suzuki and Nishimoto (2).

Egg Phospholipide II was prepared and separated into the α and β series of cephalins and lecithins by use of the methods described by Welch (9).¹

Egg Phospholipide III was prepared from egg yolk by the procedure used by Folch (13) for the preparation of brain cephalin, except that, instead of discarding the lecithins, they were recovered by pouring the absolute alcohol extract into acetone. The lecithins were fractionated into α -Lecithin III and β -Lecithin III by the method of Welch (9).¹ The cephalins were separated into α -Cephalin III and β -cephalin III by the procedure of Suzuki and Nishimoto (2).

Calf Brain Phospholipide Fractions—Calf brain phospholipide was prepared by a procedure developed in this laboratory to insure extraction of inositol and serine phospholipides as well as ethanalamine and choline phospholipides.

3 pounds of calf brain were dehydrated in a Waring blender with two successive 5.4 liter portions of acetone (400 cc. of solvent per 100 gm. of tissue) and then extracted in the same machine twice with 1 liter portions of absolute alcohol, twice with 500 cc. of petroleum ether, and once with 200 cc. of chloroform. The residue was discarded. The absolute alcohol extracts were concentrated to 200 cc. by distillation of the alcohol under reduced pressure and poured into 1 liter of acetone. The precipitate was settled by stirring, the liquid poured off, and the phospholipides blended with 200 cc. of absolute alcohol. A large portion failed to dissolve even with a second absolute alcohol treatment, and was vacuum-dried and weighed. The alcohol-soluble lecithins were reserved for combination with those obtained from the petroleum ether and chloroform extracts of brain tissue.

The petroleum ether tissue extracts were taken to dryness by distillation of the solvent at low pressure, the residue was dissolved in ethyl ether, and the material insoluble in cold ether was removed by refrigeration. The resulting ether solution (75 cc. volume) was stirred up with 150 cc. of absolute alcohol, and the precipitate was filtered at once. The filtrate was taken to dryness by vacuum distillation, and the residue was dissolved in absolute alcohol and reprecipitated by acetone. It was added to the lecithin fraction. The material insoluble in the ether-alcohol solvent was dried, weighed, and added to the cephalin fraction.

¹ Welch removed cephalin from the cadmium chloride-lecithin precipitate using alcohol-ether 7:3 and stated that this was the solvent recommended by Yoshinaga (8), whereas Yoshinaga used alcohol-ether 3:7.

On distillation of the solvent under reduced pressure, the chloroform extract of brain tissue gave a small residue insoluble in absolute alcohol and partially soluble in warm ether. The ether-soluble material was recovered by centrifugation, dried, weighed, and added to the cephalin fraction.

The combined absolute alcohol-soluble phospholipides were precipitated by acetone, filtered out, dried, and weighed. 12.69 gm. of crude lecithins were obtained. On redissolving this material in absolute alcohol, 0.462 gm. was found to be soluble only in hot absolute alcohol. It was reprecipitated by cooling the solution and was added to the cephalin fractions. The remaining lecithin weighed 12.206 gm. and was a pale amber, waxy material which gave a clear solution in either absolute alcohol or cold ether.

The amounts of cephalin recovered from each type of solvent extraction are shown below.

From absolute alcohol extraction of lecithin pptd. by acetone	1.323 gm.
" " " " " dried crude lecithins	0.462 "
" " " " " petroleum ether extract	11.805 "
" " " " " chloroform extract	1.055 "
<hr/>	
Total cephalins	14.645 gm.

The lecithins of calf brain were purified and fractionated by the procedure of Yoshinaga (8). The cephalins were separated into the α and β series by following the directions of Suzuki and Nishimoto (2).

A summary of these phospholipide fractions showing the per cent of each α fraction and each β fraction found in lecithin or cephalin prepared by the various methods is shown in Table I. As can be seen from Table I, the amounts of α and β isomers found in each lecithin or cephalin fraction varied according to the method of preparation. In the case of the lecithins, the choice of solvent for purification of the cadmium salts determines the ratio of α to β isomers. In the case of the cephalins, the method of extraction of the cephalin has the most influence.

Hydrolysis Studies

Before the lecithin and cephalin fractions prepared from calf brain and egg yolk could be analyzed, they had to be subjected to hydrolysis. A number of experiments were carried out to determine suitable conditions for this hydrolysis.

Effect of Acids and Bases on Stability of Glycerophosphates—Sodium glycerophosphate² was first analyzed for content of α isomer and then 10 mg. samples were refluxed for 1 hour on the steam bath with 10 cc. each of 1.0, 0.1, and 0.01 \times sodium hydroxide and 1.0, 0.1, and 0.01 \times sulfuric acid in

² Obtained from the Monsanto Chemical Company by Dr. Henrik Dam.

TABLE I
 α - and β -Lecithins and α - and β -Cephalins Obtained by Various Fractionations of Phospholipide from Egg Yolk and Calf Brain

Source of phospholipide	Method of preparation	Fraction	Method of fractionation	Properties used as means of separation			α and β' fractions found
				Solubility in absolute alcohol	Solubility of cadmium salt	Solubility of lead salt	
Egg yolk	Yokoyama	α -Lecithin I	Suzuki and Yokoyama	Soluble	Insoluble in ether and acetone		71.9
"		β -Lecithin "	"	"	Insoluble in ether, soluble in acetone		28.1
"	Welch	α -Lecithin II	Welch	"	Insoluble in ether-alcohol 3:7, insoluble in acetone		93.5
"		β -Lecithin "	"	"	Insoluble in ether-alcohol 3:7, soluble in acetone		6.5
"	This laboratory	α -Lecithin III	"	"	Insoluble in ether-alcohol 3:7, insoluble in acetone		95.2
"		β -Lecithin "	"	"	Insoluble in ether-alcohol 3:7, soluble in acetone		4.8
"	Nishimoto	α -Cephalin I	Suzuki and Nishimoto	Insoluble		Soluble in ether-alcohol 1:2	42.5
"		β -Cephalin "	"	"		Insoluble in ether-alcohol 1:2	57.5
"	Welch	α -Cephalin II	Welch	"		Soluble in benzene-alcohol 1:1	39.0
"		β -Cephalin "	"	"		Insoluble in benzene-alcohol 1:1	61.0
"	This laboratory	α -Cephalin III	Suzuki and Nishimoto	"		Soluble in ether-alcohol 1:2	87.1
"		β -Cephalin "	"	"		Insoluble in ether-alcohol 1:2	12.9

Calf brain	This laboratory	α -Lecithin	Yoshinaga	Soluble	Insoluble in ether-alcohol 7:3, insoluble in acetone		81.2
" "	" "	β -Lecithin	"	"	Insoluble in ether-alcohol 7:3, soluble in acetone		18.8
" "	" "	α -Cephalin	Suzuki and Nishimoto	Insoluble		Soluble in ether-alcohol 1:2	82.0
" "	" "	β -Cephalin	" "	"		Insoluble in ether-alcohol 1:2	17.1

both aqueous and 50 per cent alcohol solutions. The solutions were then adjusted to the neutral point of phenolphthalein indicator and the α -glycerophosphate and total glycerophosphate contents were determined with periodate (11). 26 per cent of the original sodium glycerophosphate was found by analysis to be present as the α isomer, and this value was not changed by heating with 0.01 N or 0.1 N base or 0.01 N acid. A decrease to 22 per cent α isomer was observed after heating with alcoholic or aqueous 1 N base, and a large increase in the α content was found after heating with 0.1 N or 1 N acid. These results agreed with the reports in the literature on the stability of glycerophosphate isomers to alkali (14-17) and the shift to a high α content in strongly acid solution (18).

Ratio of α to β Isomers Produced by Hydrolysis of Phospholipides with Acids and Bases—10 mg. samples of calf brain cephalin were hydrolyzed by heating under a reflux for 1 hour with 0.01, 0.05, 0.1, and 1 N base and 0.1 and 1 N acids, the mole ratio of reagent to phospholipide being kept constant by varying the volume of reagent used. The solutions were cooled, made acid if necessary with 0.1 N hydrochloric acid, and the fatty acids were removed by three extractions in a separatory funnel with 10 cc. portions of petroleum ether containing 5 per cent of chloroform. The aqueous layers were combined, made up to 25 cc. in a volumetric flask, and 5 cc. aliquots taken for inorganic P, α -P, and β -P (11). A similar set of experiments was carried out with egg lecithin. The results are given in Table II. An inspection of the data shows that the amount of hydrolysis in 1 hour's time varied somewhat, depending upon the strength of base or acid used, thus causing differences in the absolute amounts of each isomer obtained. With alkaline hydrolysis the ratio of α to β isomer seemed to remain constant, about 1:1, regardless of the strength of base used or of the extent of hydrolysis. With acid hydrolysis, higher amounts of α isomer were found, increasing amounts of this isomer being found with increasing strength of the acid. This is in agreement with the shift from β to α isomer with acids demonstrated with glycerophosphate salts.

Analysis of α - and β -Cephalins and α - and β -Lecithins

Phosphorus Compounds in Alkaline Hydrolysates—As a result of the experiments on the behavior of glycerophosphates with acids and bases, 0.1 N alcoholic sodium hydroxide was selected as the hydrolyzing agent for the phospholipide fractions.

10 mg. samples of each fraction were refluxed for 2 hours on the steam bath with 20 cc. of base. The hydrolysate was cooled to room temperature, made slightly acid, and chilled in ice water. The fatty acids were filtered off and washed. The combined filtrate and washings were extracted three times with petroleum ether-chloroform and then made up to 50 cc. volume.

10 cc. aliquots were taken for inorganic P, α -P, β -P, and total P (11). In addition, total P was measured on the phospholipide fractions before hydrolysis. The values found by analysis and by calculation of the α to β ratio and per cent saponification are shown in Table III.

From Table III it can be seen that egg α -Lecithin I, egg β -Lecithin I, egg α -Cephalin I, and egg β -Cephalin I, which were prepared according to the procedures used by Suzuki, Yokoyama, and Nishimoto (2-6), after alkaline hydrolysis, gave approximately 1:1 mixtures of these isomers. The same results were found in the case of the calf brain α - and β -cephalin

TABLE II

Inorganic Phosphate, α -Glycerophosphate, and β -Glycerophosphate Produced by Hydrolyzing Phospholipides with Acids and Bases for 1 Hour

Phospholipide		Hydrolyzing agent		Inor- ganic P	α -P	β -P	Ratio, α -P to β -P
	mg.		cc.	per cent	per cent	per cent	
Calf brain cephalin	10	Alcoholic 1 N NaOH	1	0.33	1.51	1.56	49:51
" " "	10	" 0.1 N NaOH	10	0.41	1.25	1.06	54:46
" " "	10	Aqueous 0.1 N NaOH	10	0.39	1.14	1.16	49:51
" " "	10	0.1 N Ba(OH) ₂	10	0.26	1.35	1.10	55:45
" " "	10	Alcoholic 0.05 N NaOH	20	0.29	1.27	1.20	52:48
" " "	10	" 0.01 " "	100	0.29	1.25	1.22	51:49
" " "	10	Aqueous 0.1 N HCl	10	0.55	1.37	0.55	71:29
" " "	10	" 1 N H ₂ SO ₄	1	0.29	1.75	1.06	62:38
Egg lecithin	9	Saturated Ba(OH) ₂	10	0.02	1.15	1.16	50:50
" " "	9	Aqueous 1 N NaOH	20	0.03	1.90	2.04	48:52
" " "	9	Alcoholic 1 N NaOH	10	0.1	1.70	2.09	45:55
" " "	9	Aqueous 0.1 N NaOH	50	0.1	1.05	1.08	47:53
" " "	36	Alcoholic 0.1 N NaOH	10	0.0	1.66	1.66	50:50
" " "	36	0.1 N Ba(OH) ₂	10	0.0	1.05	1.02	51:49
" " "	9	Aqueous 1 N H ₂ SO ₄	10	0.1	1.48	1.09	58:42
" " "	9	50% alcoholic 6 N HCl	10	0.2	2.75	0.60	82:18

fractions, which were fractionated according to the methods used by these workers for human brain. Different methods of extraction and purification did not seem to affect this ratio, since the same results were obtained in the case of egg yolk α -Lecithin II, β -Lecithin II, α -Cephalin II, and β -Cephalin II, which were prepared and fractionated according to the method of Welch (9); egg yolk α -Lecithin III and β -Lecithin III, which were prepared by the method developed in this laboratory and fractionated by the method of Welch (9); egg yolk α - and β -Cephalin III, which were prepared by the method developed in this laboratory and fractionated by the method of Suzuki and Nishimoto (2); and calf brain α - and β -lecithin, which were

prepared by the method used in this laboratory and fractionated by the method of Yoshinaga (8). These results are in marked disagreement with those obtained by Suzuki, Yokoyama, and Nishimoto (2-6) who claimed that these fractions consisted of either pure α or β isomers of the phospholipides.

Nitrogen Compounds in Acid Hydrolysates—When total nitrogen and amino nitrogen values obtained after alkaline hydrolysis of calf brain

TABLE III

Phosphorus Compounds in Alkaline Hydrolysate of α - and β -Phospholipides

Hydrolysis was accomplished by heating with 0.1 N sodium hydroxide in alcohol for 2 hours.

Phospholipide source	Fraction	Total P before hydrolysis	Phosphorus compounds found in water soluble hydrolysate					Hydrolysis
			Inorganic phosphate P	α -Glycero-phosphate P	β -Glycero-phosphate P	Total P	Ratio of α -P: β -P	
		per cent	per cent	per cent	per cent	per cent		per cent
Egg yolk	α -Lecithin I	3.96	0.00	1.78	1.76	3.81	50:50	96
" "	β -Lecithin "	3.14	0.00	1.48	1.16	2.67	56:44	85
" "	α -Cephalin "	3.62	0.00	1.36	1.47	2.81	48:52	78
" "	β -Cephalin "	4.48	0.00	1.94	2.17	4.18	47:53	93
" "	α -Lecithin II	4.08	0.03	1.53	1.64	3.50	48:52	86
" "	β -Lecithin "	4.12	0.00	1.48	1.64	3.12	47:53	76
" "	α -Cephalin "	2.44	0.16	0.63	0.79	1.65	44:56	68
" "	β -Cephalin "	Lost						
" "	α -Lecithin III	3.97	0.02	1.28	1.49	3.05	46:54	77
" "	β -Lecithin "	3.94	0.05	1.83	1.99	3.85	48:52	98
" "	α -Cephalin "	3.35	0.01	1.59	1.18	3.11	57:43	93
" "	β -Cephalin "	1.50	0.02	1.10*	0.09	1.19	93:7	79
Calf brain	α -Lecithin	4.13	0.17	1.09	1.24	2.49	47:53	60
" "	β -Lecithin	3.32	0.17	1.04	1.08	2.29	49:51	69
" "	α -Cephalin	3.60	0.11	1.50	1.78	3.40	46:54	95
" "	β -Cephalin	4.99	1.69	1.26	1.83	4.78	41:59	96

* This value is based on only one analysis and was not confirmed.

cephalin were compared with those on the intact phospholipide, a loss of nitrogen compounds was discovered. Amino nitrogen and total nitrogen before hydrolysis were 1.89 and 1.90 per cent; after hydrolysis, 0.75 and 0.82 per cent. Similar observations have been reported by Artom (19). For this reason, an acid hydrolysis of the fractions was necessary before analysis for the nitrogen constituents. Heating with 6 N hydrochloric acid in 50 per cent alcohol solution for 20 hours was found necessary to split off the fatty acids as well as to free the nitrogen bases from the gly-

erophosphoric acid. The amino nitrogen values as determined by micro periodate methods (12) agreed with those found by use of the Van Slyke nitrous acid volumetric procedure (20) only when the linkage between the nitrogen base and glycerophosphoric acid had been broken.

The fatty acids were removed by extraction with petroleum ether-chloroform and the water layers were made up to volume and aliquots taken for analysis. Amino N was determined by use of both periodate (12) and of nitrous acid (20), carboxyl N by the Van Slyke ninhydrin-CO₂ method (21), choline N by precipitation as the reineckate (22), and total N by a standard microdiffusion method (23). Total N was also determined on each fraction before hydrolysis. The analytical results are shown in

TABLE IV

Nitrogen Compounds in Acid Hydrolysates of α - and β -Phospholipides

Hydrolysis was accomplished by heating with 6 N hydrochloric acid in 50 per cent alcohol solution for 20 hours.

Phospholipide source	Fraction	Total N before hydrolysis	Nitrogen compounds found in water-soluble hydrolysate						Hydrolysis
			Choline N	Serine N	Amino N		Un-known non-amino N	Total N	
					Periodate N	Nitrous acid N			
		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Egg yolk	α -Lecithin III	1.96	1.89		0.09	0.13	None	1.90	97
" "	β -Lecithin "	2.06	1.25		0.56	0.53	"	1.80	87
" "	α -Cephalin "	1.60	0.66		0.33	0.32	0.61	1.55	97
" "	β -Cephalin "	0.50	0.0		0.46	0.49	None	0.49	98
Calf brain	α -Lecithin	2.02	0.46	0.12	0.74	0.77	0.67	1.90	94
" "	β -Lecithin	2.01	0.32	0.0	0.83	0.83	0.65	1.80	90
" "	α -Cephalin	1.56	0.17	0.66	1.32	1.41	None	1.51	97
" "	β -Cephalin	0.91	0.0	0.44	0.81	0.80	"	0.81	89
" "	Whole cephalin	1.90	0.11	0.66	1.57	1.67	"	1.78	94

Table IV. The high amino N content of most of the lecithin fractions showed that they were contaminated with cephalin. α -Cephalin from egg yolk contained considerable lecithin, as shown by its choline content. Only the β -cephalin fractions were entirely free of lecithin.

DISCUSSION

In contrast to the reported stability of the isomers of glycerophosphoric acid to heating with alkali (14-17) is the report by Bailly and Gaumé (24) that, on alkaline hydrolysis of the methyl ester of α - or β -glycerophosphate, migration of the phosphoryl group occurs, so that a mixture of two-thirds β - and one-third α -glycerophosphate is always obtained. The nearly 1:1 ratio of α - and β -glycerophosphate obtained in this work from nearly

all of the phospholipide fractions would seem to indicate that a similar situation might exist here. In this case, after the fatty acids of cephalin or lecithin have been split off, there remains a diester of phosphoric acid with glycerol and the nitrogen base. Whether an equilibrium mixture is obtained on further hydrolysis would depend on whether the nitrogen base splits off by the same mechanism as a methyl group. The hydrolysis data in this work (see Table II) would seem to indicate that the 1:1 ratio obtained is not the result of an equilibrium since, when different strengths of alkali were used to hydrolyze phospholipide samples, the strength of the alkali used or the extent of hydrolysis made no difference in the ratio of α to β isomer obtained. However, it is impossible to draw any definite conclusions on this point at the present time.

In hydrolyzing their phospholipide fractions, Suzuki, Yokoyama, and Nishimoto (2-6) heated their fractions for 3 to 6 hours with 10 per cent or saturated barium hydroxide solutions. In this work 0.1 *N* alcoholic sodium hydroxide was used for hydrolysis of the fractions because of the interference of barium with periodate methods. However, it was shown that this hydrolyzing agent gave the same ratio of isomers as either 0.1 *N* or saturated barium hydroxide solutions (see Table II). Therefore, the results obtained by us and by the Japanese workers should be comparable even if some interconversion did occur.

It is felt that the periodate methods (11) used in this work for the determination of α -P and $(\alpha + \beta)$ -P are more reliable than the double salt method used by Suzuki, Yokoyama, and Nishimoto (2-6). According to Fischer and Pfähler (25) barium α -glycerophosphate is soluble in water at 22° to the extent of only 1.3 per cent and becomes less soluble on heating. The barium nitrate double salt of β -glycerophosphate is 0.8 per cent soluble in water at 18° (7). This solubility difference is not a very satisfactory one on which to base a quantitative determination of the β salt. It would seem possible that in precipitating the barium nitrate double salt of β -glycerophosphate some of the α -glycerophosphate present might also precipitate as the barium α -glycerophosphate and thus lead to high results. In this connection, it is interesting to note that Suzuki and Nishimoto (2) hydrolyzed soy bean β -cephalin with 3 per cent sulfuric acid and found only the β isomer of glycerophosphate, contrary to the finding by Bailly (18) that heating sodium β -glycerophosphate with 2 per cent sulfuric acid converted it 87 per cent to the α isomer. It is possible that in this case Suzuki and Nishimoto mistook the barium salt of α -glycerophosphate for the barium nitrate double salt of β -glycerophosphate.

It was impossible to prepare pure lecithins or cephalins by the methods employed in this work. In the purest preparations obtained, egg lecithin and brain cephalin, the nitrogen and phosphorus values of the α fraction

approached those generally accepted for lecithin and cephalin. In general, the composition of the α fractions was more constant, while that of the β fractions varied considerably because the impurities seemed to be concentrated in these latter fractions.

The α - and β -phospholipide fractions are reproducible solubility fractions, but their differences in solubility seem to be caused by something other than the type of glycerophosphate present. In the case of the lecithins, it may possibly be the amount of cephalin impurity present, whereas in the case of the cephalins it may be the presence of serine or inositol phospholipide which differs in solubility and attraction toward metallic ions from ethanol-amine phospholipide. Calf brain β -cephalin, especially, seems to bear some resemblance to the inositol phospholipide described by Folch and Woolley (26). A further comparison of α - and β -cephalins with fractions prepared by the procedure of Folch (13) will be described in a later paper.

SUMMARY

The observations by previous workers that isomers of glycerophosphate are stable in alkaline solution and labile in acid solution were confirmed.

Cephalins and lecithins were prepared from egg yolk and calf brain and fractionated into the α and β series.

The phosphorus compounds were analyzed after alkaline hydrolysis and the nitrogen compounds after acid hydrolysis.

The α - and β -lecithins and α - and β -cephalins, instead of being pure isomers as reported in the literature, were found to contain approximately equal amounts of α - and β -glycerophosphate. The solubility differences observed are not due to isomerism of the glycerophosphates.

The β -cephalin fraction of calf brain resembled closely in appearance and solubility the inositol phospholipide described by Folch and Woolley (26).

The author wishes to acknowledge his great indebtedness to Dr. W. R. Bloor for making this work possible.

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A COMPARISON OF BRAIN CEPHALIN FRACTIONS*

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In a previous paper,¹ α - and β -lecithins and α - and β -cephalins of egg yolk and of calf brain were shown to contain approximately equal proportions of α - and β -glycerophosphates instead of the pure isomers as reported by Suzuki, Yokoyama, and Nishimoto (2-7). The α -phospholipide fractions were found in general to have the properties usually attributed to lecithin and cephalin, while the β -fractions varied in composition with the source of phospholipide and methods of purification. The β -cephalin fraction of calf brain was similar in appearance and solubility to the inositol phospholipide described by Folch and Woolley (8) and Folch (9).

Folch (9) described a fractionation procedure by which inositol phospholipide, phosphatidyl serine, and phosphatidyl ethanolamine were precipitated in that order by addition of increasing amounts of absolute alcohol to a chloroform solution of ox brain cephalin. In this paper the results of chemical analysis of fractions prepared in this manner from calf brain cephalin and of the products of their acidic and basic hydrolysis are compared with those previously obtained¹ for the α - and β -cephalin fractions.

EXPERIMENTAL

Analytical Methods—Inorganic P, α -glycerophosphate P, β -glycerophosphate P, and total P were determined by the microcolorimetric methods described in a previous paper (10). Periodate N (11), a measure of the nitrogen present as free serine and free ethanolamine, was compared with amino N by the Van Slyke volumetric procedure (12). Serine N was measured by the Van Slyke *et al.* ninhydrin titrimetric method (13). Choline was precipitated as the reineckate and measured colorimetrically (14). Total N was measured as the ammonia recovered by microdiffusion after digestion with sulfuric acid, potassium sulfate, and copper-selenium catalyst (15). Scherer's test for inositol was used to indicate its presence in the fractions.

* The data in this paper are taken from a thesis submitted to the Graduate School of The University of Rochester in partial fulfilment of the requirements for the degree of Doctor of Philosophy. A preliminary report of these data has been given (1).

¹ Burmaster, C. F., *J. Biol. Chem.*, 165, 565 (1946).

Preparation of Chloroform-Alcohol Fractions of Calf Brain Cephalin—Cephalin was prepared from 520 gm. of calf brain by the procedure described by Folch (9). In this procedure the tissue is first dehydrated with acetone, then the lecithins are extracted with alcohol, and finally the cephalins extracted by petroleum ether are purified by refrigeration of a cold ethyl ether solution and precipitation of the ether solution in alcohol. Folch (9) used only the cephalin from the petroleum ether extract for the subsequent fractionation. In our work 2.70 gm. of cephalin were found in the alcohol extract of brain and 2.77 gm. in the petroleum ether extract. Both cephalin preparations were purified and fractionated by addition of alcohol to chloroform solutions but only the fractions of cephalin from the petroleum ether extract are reported here, since scarcely any inositol phospholipide was found in cephalin from the alcohol extracts.

TABLE I

Comparison of Brain Cephalin Fractions Obtained by Chloroform-Alcohol Method

Fraction No.	Prepared from calf brain cephalin			Reported by Folch (9) for ox brain cephalin			Reported by Rathmann (16) for pig brain cephalin		
	Yield per gm. cephalin	Total P	Total N	Yield per gm. cephalin	Total P	Total N	Yield per gm. cephalin	Total P	Total N
	gm.	per cent	per cent	gm.	per cent	per cent	gm.	per cent	per cent
I	0.1669	4.18	1.60	0.22	4.25	1.15	0.19	3.96	1.47
II	0.3519	3.60	1.62	0.10	3.86	1.36			
III	0.1884	3.70	1.51	0.27	3.58	1.62	0.21	3.80	1.61
IV	0.0833	3.45	1.78	0.08	3.60	1.75			
V	0.2085	3.34	1.76	0.15	3.65	1.78	0.18	3.65	1.79

Comparison of Chloroform-Alcohol Fractions of Calf Brain Cephalin with Those Obtained from Ox Brain and Pig Brain Cephalin—The weights of the fractions produced from 1 gm. of calf brain cephalin and the total N and total P values obtained by chemical analysis are compared in Table I with the results reported on chloroform-alcohol fractionations of ox brain cephalin by Folch (9) and of pig brain cephalin by Rathmann (16). As can be seen, except for the high nitrogen values in Fraction I of pig brain and Fractions I and II of calf brain, the fractions obtained by both Rathmann (16) and us correspond very closely to those obtained by Folch (9). These higher nitrogen values may be explained by the fact that neither the fractions prepared by Rathmann nor those prepared by us were purified by dialysis with distilled water before analysis as were those prepared by Folch.

Hydrolysis of Chloroform-Alcohol Fractions of Calf Brain Cephalin—It has been shown¹ that an alkaline hydrolysis of phospholipide is necessary

before analysis of the glycerophosphate isomers and an acid hydrolysis before analysis of the nitrogen bases (17).¹ The alkaline hydrolysis was carried out by heating 10 mg. samples for 2 hours with 20 cc. of 0.1 N sodium hydroxide in 50 per cent alcohol. The acid hydrolysis was carried out by heating 10 mg. samples for 24 hours with 20 cc. of 6 N hydrochloric acid in 50 per cent alcohol. The fatty acids and unsaponified material were removed from all of the hydrolysates by three extractions of the acidified solution with petroleum ether containing 5 per cent of chloroform. The

TABLE II
Analysis of Hydrolysates of Calf Brain Cephalin Fractions

Fraction No.	Found after alkaline hydrolysis						Found after acid hydrolysis						
	Inorganic P	α -Glycero-phosphate P	β -Glycero-phosphate P	$\frac{\alpha}{\beta}$ -P	Total P	Hydrolysis*	Choline N	Serine N	Ethanolamine N†	Amino N		Total N	Hydrolysis†
										Periodate N	NH ₂ -N (Van Slyke)		
	per cent	per cent	per cent		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
I	1.34	0.86	1.30	40:60	3.93	94	0.03	0.87	0.72	0.76	1.59	1.62	101
II	0.52	0.98	2.00	33:67	3.56	99	0.02	0.84	0.77	1.45	1.61	1.58	98
III	0.38	1.25	1.65	43:57	3.62	98	0.02	1.13	0.35	1.47	1.48	1.49	99
IV	0.22	1.17	1.53	43:57	3.20	93	0.16	0.51	1.19	1.61	1.70	1.80	101
V	0.18	0.96	1.32	42:58	2.55	76	0.05	0.0	1.05	1.04	1.05	1.12	64
α -Ceph- alin	0.11	1.50	1.78	46:54	3.40	95	0.17	0.66	0.66	1.32	1.41	1.51	97
β -Ceph- alin	1.69	1.26	1.83	41:59	4.78	96	0.00	0.44	0.37	0.81	0.80	0.81	89

* Calculated from the ratio of the total P in the hydrolysate and in the whole fraction.

† Calculated by subtracting serine N from NH₂-N (Van Slyke).

‡ Calculated from the ratio of the total N in the hydrolysate and in the whole fraction.

extracted aqueous solutions were then diluted to 25 cc. in volumetric flasks and aliquots were taken for analysis of the phosphorus and nitrogen compounds.

Results

Analysis of Fractions Obtained by Chloroform-Alcohol Method from Calf Brain Cephalin—The results of chemical analysis of the products of alkaline and of acid hydrolysis of Fractions I to V are compared in Table II with those reported previously¹ for the α -cephalin and β -cephalin fractions. Folch (9) found that inositol phospholipide was concentrated in Fraction I,

phosphatidyl serine in Fraction III, and phosphatidyl ethanolamine in Fraction V. This was confirmed here by the high inorganic P and total P

TABLE III

Comparison of Properties of Brain β -Cephalin and of Inositol Phospholipides

Source	Fraction	Prepared by	N	P	Mole ratio, N:P	NH ₂ -N	COOH-N	Inositol
			<i>per cent</i>	<i>per cent</i>		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Calf brain	β -Cephalin*	Burmester†	0.91	4.99	1:2.5	0.81	0.44	Present
" "	Cephalin, chloroform-alcohol Fraction I†	" (this paper)	1.60	4.18	1:1.2	1.59	0.87	"
" "	Inositol phospholipide§	" "	1.04	4.76	1:2.1			"
Ox brain	Cephalin, chloroform-alcohol Fraction I	Folch (9)	1.15	4.25	1:1.7	1.15	0.70	6.8
Pig "	" "¶	Rathmann (16)	1.47	3.96	1:1.2			
Beef brain	Inositol phospholipide	Folch and Woolley (8)	1	4.5	1:2			6.8-8.6
Soy bean	Lipositol**	Woolley (18)	0.98	3.1	1:1.4	0.80	None	16
" "	Inositol phospholipide	Klenk and Sakai (20)	0.69	3.25	1:2.1	0.37		Present

* When dry, insoluble in all lipid solvents. When moist, soluble in chloroform; less soluble in benzene, ether; insoluble in petroleum ether, glacial acetic acid, alcohol.

† Burmaster, C. F., *J. Biol. Chem.*, **165**, 565 (1946).

‡ Soluble in chloroform, less soluble in benzene, ether; insoluble in petroleum ether and chloroform-alcohol, 1:1.

§ When dry, insoluble in all lipid solvents. When moist, soluble in chloroform; less soluble in benzene, ether; insoluble in petroleum ether, glacial acetic acid, ether-alcohol, 1:1.

|| Soluble in chloroform; insoluble in chloroform-alcohol, 1:1.135.

¶ Soluble in chloroform, moist ether; insoluble in ethanol, dry ether, petroleum ether, glacial acetic acid.

** When dry, insoluble in all solvents tried. When moist, soluble in chloroform, benzene, ether; insoluble in alcohol, glacial acetic acid.

in Fraction I, the fact that nearly all of the amino N in Fraction III was serine N, and by the absence of serine N in Fraction V. When tested qualitatively, the hydrolysates of Fractions I, II, III, and β -cephalin were found to contain inositol. Woolley (18) found that the nitrogen bases of

inositol phospholipide from soy bean were not freed by acid hydrolysis. The low periodate N results obtained in Fractions I and II compared to amino N show that this observation is also true of inositol phospholipide of calf brain. The high inorganic P and total P contents of hydrolyzed β -cephalin show its similarity to fractions rich in inositol. All of the nitrogen values for β -cephalin were lower than those obtained for Fraction I but the relative amounts of serine N and ethanolamine N were nearly the same. The lower nitrogen content of the β -cephalin fraction may be due to the method used to recover it from the lead salt.¹ The emulsification of a chloroform suspension of the lead salt with 1 per cent hydrochloric acid may remove some of the nitrogen bases as well as the lead. The α -cephalin fraction contained both serine and ethanolamine but only a trace of inositol.

Comparison of Inositol Phospholipide with β -Cephalin Fraction—The similarity of Fraction I and β -cephalin led to an investigation of the solubility of inositol phospholipide and its lead salt in the solvents used for the separation of α - and β -cephalin fractions (2, 5, 6, 19).¹ Inositol phospholipide was prepared from the remainder of Fraction I by precipitation of chloroform solutions with dry methanol and absolute alcohol as described by Woolley (18) for the preparation of lipositol. It was soluble in chloroform, less soluble in benzene or ether, and insoluble in petroleum ether and glacial acetic acid. A saturated solution in benzene or ether was completely precipitated by addition of less than an equal volume of alcohol or of a solution of basic lead acetate in alcohol. The lead salt was insoluble in all organic solvents tried. When it was suspended in chloroform and emulsified with a 1 per cent solution of hydrochloric acid, a precipitate of lead chloride was formed and the free phospholipide was soluble in the chloroform layer. In these respects the behavior of inositol phospholipide was identical with that of the β -cephalin fraction. The results of chemical analysis of this inositol phospholipide prepared from Fraction I showed even closer agreement with those for β -cephalin than did those for Fraction I. This comparison and a summary of the values reported in the literature for inositol-rich phospholipides are shown in Table III.

DISCUSSION

It was felt that inositol phospholipide might cause error in the determination of β -P, since it has been shown¹ that if unsaponified phospholipide is not extracted from the hydrolysate it is split during the conversion of β -glycerophosphate to inorganic P by hot acid periodate and causes high results. Inositol phospholipide was found to be soluble in chloroform but not very soluble in the petroleum ether-chloroform mixture used to remove fatty acids and unsaponified phospholipide from the hydrolysates. When

the alkaline hydrolysate of Fraction I was extracted with chloroform in addition to the petroleum ether-chloroform extractions and then analyzed again for β -P, no decrease in β -P was found. This shows that the high β -P results were not due to the presence of unsaponified inositol phospholipide.

In a previous paper,¹ the possibility was mentioned that the ratios of α - to β -glycerophosphate found might be the result of an equilibrium produced by the hydrolyzing agent, as shown in the case of the methyl ester of glycerophosphoric acid by Bailly and Gaumé (21). Although at that time what little evidence was available seemed to be against the idea of an equilibrium, there is still no conclusive proof either way. During the course of this work, a sample of phosphatidyl serine was prepared by reprecipitation of Fractions II and III from chloroform with alcohol, and the ratio of α - to β -P was determined after hydrolysis with 0.1 N sodium hydroxide for 2 hours. The α to β ratio was found to be 22:78 which is in close agreement with values obtained by Folch (22) after hydrolysis with barium hydroxide. The difference between this ratio for purified phosphatidyl serine and those obtained for the other fractions after the same alkaline hydrolysis is difficult to explain if one accepts the theory of an equilibrium produced by the hydrolysis. However, it may be that these differences are caused by the varying rates at which the nitrogen compounds are split from the glycerophosphate and that a more prolonged hydrolysis might lead to the same α and β ratio for all the phospholipides. Further work is being done on the question of this equilibrium.

The close agreement of the data for inositol phospholipide, especially when purified, with that of β -cephalin would seem to establish definitely the identity of the β -cephalin fraction. With the α -cephalin fraction, while it has been shown that it contains serine and ethanolamine phospholipides, there is not this striking agreement in analytical data with any one fraction. However, it was found that there was considerable cephalin (about one-half the total) in the alcohol extract of brain which was discarded by Folch (9). This cephalin consisted almost entirely of phosphatidyl serine and phosphatidyl ethanolamine and had an α to β ratio of 49:51. When this ratio was averaged with the values for Fractions III, IV, and V, a ratio identical with that of α -cephalin was obtained.

SUMMARY

A comparison of the solubilities and chemical analyses of α - and β -cephalin and chloroform-alcohol cephalin fractions from calf brain shows that inositol phospholipide is the major constituent of the β -cephalin fraction and that phosphatidyl serine and phosphatidyl ethanolamine are major constituents of the α -cephalin fraction.

The author wishes to acknowledge his great indebtedness to Dr. W. R. Bloor for making this work possible.

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INTRACELLULAR DISTRIBUTION OF ENZYMES

I. THE DISTRIBUTION OF SUCCINIC DEHYDROGENASE, CYTOCHROME OXIDASE, ADENOSINETRIPHOSPHATASE, AND PHOSPHORUS COMPOUNDS IN NORMAL RAT TISSUES

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The study of the enzymatic and chemical properties of the particulate components of the cell represents a problem of paramount importance because it permits an integration of two important fields of science, cytology and biochemistry. Functions that have been assigned to cellular structures as the result of cytological observations can now be investigated by isolation of the particulate cellular components and examination of their chemical and biochemical properties.

The first steps in this direction were taken by Bensley and his coworkers (2, 3, 10, 11), by Claude (4, 5, 7), and by Dounce (8), who developed centrifugation techniques for the isolation of mitochondria, microsomes, chromatin threads, and nuclei. Enzyme studies on isolated mitochondria were made by Lazarow and Barron (12) and were extended by Claude and his associates to include the microsomes and a larger variety of enzymes (6). The enzyme properties of the largest particulate component of the liver cell, the nucleus, have been investigated by Dounce (8).

In studying the distribution of enzymes in tissue fractions, it is necessary to measure the enzyme content of each of the fractions as well as that of the original tissue. It is not a valid procedure to discard a tissue fraction and calculate its enzyme content later by subtracting the enzyme contents of the other tissue fractions from that of the original tissue since the possibility of the presence of an unknown cofactor or an inhibitor in the original tissue suspension cannot be excluded unless the activity of all fractions is measured and shown to equal the activity of the original tissue. This principle has been carefully followed in the present work not only with respect to enzyme assays but also in the measurement of nucleic acids and other phosphorus compounds in the isolated tissue fractions.

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Methods and Materials

Tissue Preparation—The tissues were removed as rapidly as possible from rats killed by decapitation and were homogenized in 9 volumes of ice-cold alkaline water¹ (pH 9.5 to 10.0; see (4)) in the apparatus of Potter and Elvehjem (17).

Tissue Fractionation—The tissue homogenates were fractionated essentially as described by Claude (4, 5). The procedure was as follows: 10 ml. of a 10 per cent homogenate were centrifuged at $1500 \times g$ to sediment the nuclei and whole cells. Mitochondria were also sedimented at this point but the loss could be minimized by resuspending the sediment in 2.5 ml. of alkaline water with a loose homogenizer and then recentrifuging at the same speed.¹ This washing procedure was repeated three times. The washed sediment was made up to 10 ml. with water and termed the nuclear fraction. The supernatant and washings from the nuclear sediment were combined and centrifuged at $2400 \times g$ to sediment the mitochondria or large granules.² The mitochondria were washed three times at the same speed after suspension in 5.0 ml. of alkaline water. The washed mitochondria were made up to 10 ml. and the supernatant and washings from the mitochondria were combined, made up to 30 ml., and termed the unfractionated residue.

Cytological Methods—The original homogenate and the tissue fractions were examined in both the bright and in the dark-field microscopes. Smears were also made and stained with aniline-acid fuchsin-methyl green (1).

Enzyme Assays—Succinic dehydrogenase, cytochrome oxidase, and adenosinetriphosphatase were measured by methods described previously (9, 20). The assays were made simultaneously on the original homogenate and on the tissue fractions as soon as the fractionation was completed. The time which elapsed between the killing of the animal and the beginning

¹ Alkaline water was used instead of isotonic NaCl because it was found that the latter resulted in the accumulation of 50 per cent or more of the original succinoxidase activity in the nuclear sediment. Washing with saline did not reduce the succinoxidase activity of the nuclear sediment. Alkaline water also led to an accumulation of succinoxidase activity in the nuclear fraction but in this case it was possible to reduce the succinoxidase content to 5 per cent of the original by washing with water, as described in the text. The loss of succinoxidase activity in the nuclear sediment was paralleled by losses of PNA, dry material, lipid phosphorus, etc. Thus it would seem that the accumulation of these substances in the nuclear fraction was explainable on the basis of a sedimentation of large granules.

² The terms "large granules" and "mitochondria" are used interchangeably in this paper, but with the understanding that the former is probably a more correct term (in the case of liver), since secretory granules as well as mitochondria are present in this fraction (see (5)).

of the assays was 4 to 5 hours. In some cases only 1 enzyme was measured and in others all 3 enzymes were measured at the same time.

Analytical Methods—After the enzyme assays were completed, the remaining portions of the original homogenate and of the tissue fractions were frozen in liquid air and stored overnight. After thawing, acid-soluble phosphorus, phospholipide, "phosphoprotein," and nucleic acids were determined as described previously (18). In addition nitrogen was determined on the "phosphoprotein" fraction by the method of LePage and Umbreit (13). Dry weight was determined on aliquots of the tissue suspension by drying *in vacuo* over P_2O_5 in tared bottles.

Results

The results of the enzyme assays and the analytical results are presented in Tables I and II. The results are expressed in terms of 1.0 ml. of *original* 10 per cent homogenate (equivalent to 100 mg. of fresh tissue) so that the amounts of materials found in the tissue fractions could be compared with the amounts found in the original homogenate. It is evident that the values obtained on the tissue fractions agreed well with the results obtained with the whole homogenate (within about 5 per cent). It should be pointed out here that such excellent agreements were not the result of averaging several experiments but were observed in each experiment.

Cytological Results—Examination of the nuclear fraction revealed numerous intact nuclei which had clumped together in large masses as well as some whole cells and mitochondria. The mitochondria fraction contained numerous particles of fairly uniform size which stained bright red with Bensley's aniline-acid fuchsin-methyl green (1). No whole cells or nuclei were visible in this fraction or in the unfractionated residue. The unfractionated residue was found to contain some mitochondria and numerous fat globules.

Enzyme Assay Results—It is apparent from Tables I and II that a large portion of the original enzyme activity of rat liver and kidney was localized in the large granule fraction. This was especially true of succinic dehydrogenase and cytochrome oxidase, since 70 per cent or more of the enzyme activity of the original homogenate was found in this fraction. Adenosine-triphosphatase was more generally distributed, although most of the enzyme activity was again associated with the mitochondria. The fact that all of the enzyme activity present in the original homogenate could be recovered in the three tissue fractions is highly indicative of the validity of the assay procedures. This statement is especially applicable to cytochrome oxidase, since the results obtained on the whole homogenate and on each of the tissue fractions must be corrected for the autoxidation of ascorbic acid (see (20)). It is also interesting to note that the cytochrome oxi-

TABLE I
Distribution of Enzymes, Phosphorus-Containing Compounds, "Protein" Nitrogen, and Dry Material
in Fractions of Rat Liver Homogenates

The average values are given in bold-faced type.

Tissue fraction	Enzyme activities			Phosphorus distribution per 100 mg. fresh tissue						"Protein" nitrogen per 100 mg. fresh tissue	Dry material per 100 mg. fresh tissue
	Succin-oxidase*	Cytochrome oxidase*	Adenosine-triphosphatase†	DNA†	PNA†	Nucleic acid	Acid-soluble	Lipoid	"Protein"		
Original homogenate	383 (337-424)	1012 (880-1180)	865 (754-940)	22.6 (18.8-29.7)	65.2 (62.4-70.1)	95 (82-108)	125 (116-144)	155 (111-207)	40.2 (25.7-59.5)	1970 (1500-2305)	31.9 (29.5-33.4)
Nuclear fraction	25.4 (22.2-46.5)	54.6 (44-65)	231 (134-261)	23.4 (18.3-30.6)	4.9 (1.9-7.4)	27.2 (20.3-33.5)	4.8 (2.7-6.5)	19.0 (14.5-27.3)	6.1 (3.4-8.8)	197 (124-227)	3.3 (3.1-4.0)
Mitochondria fraction	289 (218-385)	748 (660-815)	416 (305-425)		11.4 (9.3-16.5)	16.0 (12.7-21.0)	4.9 (3.7-6.3)	42.1 (32.8-55.1)	11.4 (8.4-14.0)	434 (297-795)	5.7 (4.3-5.8)
Unfractionated residue	45.5 (30.0-54.8)	147 (143-150)	257 (231-300)		47.8 (41.3-53.7)	61.5 (49.4-75.0)	115 (96-131)	95.0 (75-120)	19.7 (13.4-25.0)	1250 (900-1580)	23.3 (21.2-26.4)
No. of measurements	6	3	3								8

* The activities are expressed as c.mm. of O₂ taken up per 10 minutes by the equivalent of 100 mg. of fresh tissue.

† The activities are expressed as micrograms of phosphorus liberated in 15 minutes by the equivalent of 100 mg. of fresh tissue.

‡ Phosphorus calculated from pentose measurements.

dase activity of the homogenate and of the fractions is always in excess of the succinoxidase activity, thus indicating that the succinoxidase assay is a valid measure of succinic dehydrogenase.

It is difficult to decide whether the succinic dehydrogenase and cytochrome oxidase activities observed in the nuclear sediment and in the unfractionated residue are integral properties of the fractions or whether the activities are due to contamination of the fractions by large granules. The latter appears to be the more likely explanation in view of the cytological observations and because of the high concentrations of these enzymes in the large granule fraction.

Analytical Results—Perhaps the finding of greatest interest is the fact that all of the desoxyribose nucleic acid (DNA) in the original homogenate

TABLE II

Distribution of Succinoxidase, Phosphorus-Containing Compounds, "Protein" Nitrogen, and Dry Material in Fractions of Rat Kidney Homogenates

Each figure represents the average of two experiments.

Tissue fraction	Succinoxidase activity*	Phosphorus distribution per 100 mg. fresh tissue						"Protein" nitrogen per 100 mg. fresh tissue	Dry material per 100 mg. fresh tissue
		DNA*	PNA*	Nucleic acid	Acid-soluble	Lipoid	"Protein"		
		γ	γ	γ	γ	γ	γ	γ	mg.
Original homogenate.....	647	37.4	31.8	67.0	94	117	36.0	1600	25.0
Nuclear fraction ..	49	37.4	3.4	34.8	3.4	14.5	7.8	218	4.1
Mitochondria fraction.....	447		4.2	7.4	4.6	33.0	14.5	308	4.2
Unfractionated residue.....	126		23.8	29.0	85.9	71.3	15.5	890	17.6

* See Table I.

is found in the nuclear fraction. This lends excellent support to the cytological observations and also to the theory that this nucleic acid is found only in the nucleus of the cell. It should be pointed out that the DNA content of the nuclear fraction of rat liver is 7.0 per cent in contrast to the 12 to 20 per cent reported by Dounce for pure nuclei (8). Several reasons might be mentioned in explanation of this discrepancy. In the first place our nuclear fraction is admittedly contaminated by the large granule fraction. Another source of contamination of this fraction may be ground glass produced during the homogenization. A further possibility is that the nuclei isolated by Dounce had a higher nucleic acid content than did those which were lost during the purification process.

Pentose nucleic acid (PNA) was found in each of the three fractions, although most of it was found in the unfractionated residue. In the case of

the liver, the PNA distribution closely paralleled the distribution of dry material in each of the fractions. The question of whether PNA is an integral constituent of the nucleus is of considerable importance and is one which requires similar considerations to those mentioned in regard to whether the oxidative enzymes were associated with the nucleus or present as contaminants. In addition to these considerations it must be recalled that the PNA measurements in the nuclear fraction involve considerable difficulty, since the measurement of PNA is subject to a correction factor in the presence of a large amount of DNA (see (18)).

A further point of interest is the large amount of lipoid phosphorus found in the large granule fraction of liver and kidney. This supports the findings of Claude (5) and is in harmony with the idea that the large granules are phospholipoprotein complexes.

DISCUSSION

The results of the tissue fractionation clearly show that the succinic dehydrogenase and cytochrome oxidase activities are associated with the large granules. These findings are in harmony with those reported by Claude (6). It must be emphasized, however, that the present experiments differed from those of Claude in that all of the tissue fractions were examined for enzymatic activity. Claude on the other hand discarded the nuclear fraction and based his enzyme results on the nuclei-free extract so produced. From the considerations presented in the introduction, it is obvious that such a procedure would make it difficult to correlate the data on the extract with that on the original tissue. The situation is further complicated by the fact that large losses of enzyme can occur in the nuclear sediment unless proper precautions are taken.¹

The present data³ and the data of Claude (6) are in marked contrast to the results of Lazarow and Barron (12) who found that the succinoxidase activity of the nuclear concentrate was higher than that of either the original tissue or the mitochondria fraction. The high activity of the nuclear fraction can probably be accounted for by the presence of large numbers of whole cells in this fraction (see (15)). Their data on the whole liver and on the nuclear and mitochondria fractions are to be regarded with considerable

¹ Data obtained in this laboratory by Potter in 1942 are also in complete agreement with those presented herewith, and are in contrast to those presented by Lazarow and Barron. This work was done prior to the development of the author's methods for nucleic acid analyses and was not published because the chemical proof that nuclei and mitochondria had been separated was not available. Microscopic examination revealed the nuclei in the nuclear fraction, but it was felt that the absence of stainable nuclei from the mitochondria fraction was not sufficient proof that fragments were not present.

suspicion, since no attempt was made to saturate the system with cytochrome *c*, a known component of the succinoxidase system (14, 15, 20).

In studying the intracellular distribution of enzymes it is important to know (1) the proportion of the total enzyme activity accounted for by a particular cell fraction and (2) whether the enzyme is more concentrated in any fraction than it is in the original tissue. It is conceivable that a large portion of the enzyme activity of the original tissue could be present in a given fraction and still not be concentrated in that fraction. An answer to these questions can be obtained by expressing the data in two ways. In the first method, which was used in Tables I and II, the enzyme activity of the tissue fractions is expressed in terms of 100 mg. of original tissue. This method shows how much of the original enzyme activity is present in each

TABLE III

Cytochrome Oxidase Activities of Rat Liver and Rat Liver Fractions Expressed on Original Wet Weight Basis and on Dry Weight Basis

Tissue fraction	Cytochrome oxidase activity			
	O ₂ uptake per 100 mg. original tissue per 10 min.*	Fraction of original activity	O ₂ uptake per mg. dry material per 10 min.†	Fraction of original activity
	<i>c.mm.</i>	<i>per cent</i>	<i>c.mm.</i>	<i>per cent</i>
Original homogenate	1012	100	31.5	100
Nuclear sediment.....	54.6	5.4	16.6	52.7
Large granule fraction. .	748	73.9	131	416
Unfractionated residue ...	147	14.5	6.3	20.0

* Taken from Table I.

† Calculated from Table I.

tissue fraction and enables one to add the activities of the fractions and compare the total with the activity of the original tissue. In the other method the enzyme activity per mg. of dry material is calculated for each fraction as well as for the original tissue. If a concentration of the enzyme has been achieved, the activity of the tissue fraction will be greater when expressed in the latter terms than will the activity of the original tissue. The cytochrome oxidase activities of the liver fractions have been calculated by the two methods and are presented in Table III. The results show that a large proportion of the original enzyme activity (73.9 per cent) was associated with the large granule fraction and that a 4-fold concentration of the enzyme was achieved by the separation of this fraction. On the other hand, the data show that only a small portion of the original enzyme activity was associated with the nuclear fraction and with the unfractionated residue and that the enzyme was less concentrated in these fractions

than it was in the original tissue. The results on the nuclear fraction are in agreement with those of Dounce (8) who reported enzyme activity per mg. of dry material. He found that the cytochrome oxidase activity of nuclei was 50 to 60 per cent as great as that of the original tissue. The present results emphasize that, although the activity of the nuclear fraction was 52.7 per cent as great as that of the original tissue, only 5.4 per cent of the original enzyme activity was present in this fraction. Thus the need for determining the enzyme activity in all tissue fractions obtained has again been emphasized and the value of expressing the enzyme activities in terms of the original wet tissue and in terms of the dry material present has been demonstrated.

The demonstration of the association of enzymes involved in carbohydrate metabolism with the large granules suggests that the latter play an important rôle in cellular reactions. The significance of the association of these enzymes with granules containing PNA must remain unsolved for the present, since evidence which suggested that these enzymes were ribonucleoproteins (16) can apparently be explained on another basis (19).

SUMMARY

1. Rat liver and kidney homogenates were separated by centrifugation into a nuclear fraction, a mitochondria or large granule fraction, and an unfractionated residue.

2. The original homogenate and each of the fractions were assayed for succinic dehydrogenase, cytochrome oxidase, and adenosinetriphosphatase, and were analyzed for desoxypentose and pentose nucleic acids, acid-soluble, lipid, and "protein" phosphorus, "protein" nitrogen, and dry material.

3. The major part of the activity of the enzymes tested was found to be associated with the large granule fraction.

4. Desoxypentose nucleic acid was found only in the nuclear fraction. Pentose nucleic acid was found in all three fractions but most of it was present in the unfractionated residue.

5. Lipid phosphorus was present in higher concentrations in the large granule fraction, although the largest portion was in the unfractionated residue. Each of the other components measured was present mainly in the unfractionated residue.

6. These results were discussed in relation to those of previous investigators.

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COMPARISON OF THE METABOLISM OF AMMONIA AND MOLECULAR NITROGEN IN AZOTOBACTER*

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An earlier report (1) dealing with the distribution of isotopic nitrogen in cells of *Azotobacter vinelandii* fixing molecular nitrogen provided evidence that ammonia might be the initial stable form of nitrogen in the fixation process. This conclusion was based on the observation that the N^{15} supplied this organism accumulated in the glutamic acid fraction, as had been already observed in higher plants (2) and animals (3) supplied ammonium labeled with the heavy nitrogen isotope. Comparable studies in which ammonium containing N^{15} was given to *Azotobacter vinelandii* are reported here.

EXPERIMENTAL

The details of the methods used to grow the cells are given in the previous paper (1). In Experiment 1, 6 liter cultures of *Azotobacter vinelandii* were grown in 10 liter Pyrex bottles aerated vigorously with bacteria-free air. After 18 hours at 30° each culture was supplied with 20 mg. of ammonium sulfate nitrogen (3.33 parts per million N) containing 33 atom per cent N^{15} excess. After 15 minutes aeration in the presence of the ammonium ion, the culture was quickly passed through a Sharples supercentrifuge and the cells removed and placed in boiling 8 N sulfuric acid. On the average 12 minutes elapsed from the cessation of aeration until the cells were placed in boiling acid. Cells from eleven 6 liter cultures were combined and hydrolyzed for 36 hours in 8 N sulfuric acid under a reflux.

In Experiment 2 two 6 liter cultures were grown as described, and 5 p.p.m. of N^{15} -enriched ammonium sulfate nitrogen were added when the cultures were $19\frac{1}{2}$ hours old. 3 and 8 minutes after the addition of the N^{15} one culture was mixed with 60 ml. of 6 N sulfuric acid. The cells were recovered and placed in boiling 8 N sulfuric acid about 15 minutes after the addition of the 6 N acid; they were hydrolyzed under a reflux with 8 N sulfuric acid. Hereafter reference will be made to the 3, 8 (Experiment 2), or 15 minute (Experiment 1) cultures, hydrolysates, fractions, or compounds.

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A fractionation essentially the same as described previously (1) was employed. Humins were removed by filtration and the sulfate ion was removed with $\text{Ba}(\text{OH})_2$. The amino acids of the 15 minute hydrolysate (Experiment 1) were precipitated with Neuberg's reagent; the solution recovered from the decomposition of the Neuberg precipitate, from which isolations were made, contained 3580 mg. of N with 0.899 atom per cent N^{15} excess. The 3 and 8 minute hydrolysates (Experiment 2) were not treated with Neuberg's reagent; the total 3 minute hydrolysate contained 681 mg. of N with 0.174 atom per cent N^{15} excess, and the 8 minute hydrolysate 787 mg. of N with 0.354 atom per cent N^{15} excess.

Arginine—With the 15 minute hydrolysate, arginine flavanate was separated directly from the decomposed Neuberg filtrate, whereas arginine flavanate was recovered after removal of the 3 and 8 minute dicarboxylic acids in the other hydrolysates. Portions of the arginine flavanates, purified by recrystallization, were decomposed with cold, concentrated hydrochloric acid, and the residual flavanic acid in the filtrates was removed with activated carbon. The regenerated arginine solutions were analyzed for N^{15} . The *p*-toluenesulfonylarginine derivative was prepared only from the 8 minute regenerated arginine. The melting point observed was 258° ; nitrogen found 16.84 per cent; theory 17.07 per cent.

Dicarboxylic Amino Acids—The barium salts of the dicarboxylic acids were precipitated in alcoholic solution. After removal of barium, concentration of the filtrate, and saturation with hydrogen chloride gas, glutamic acid hydrochloride was obtained. The 3, 8, and 15 minute derivatives melted at 203° , 202° , and 204° respectively (204° accepted), and contained 7.54, 7.52, and 7.49 per cent nitrogen; theory, 7.63 per cent.

Copper aspartate was prepared from the filtrates from glutamic acid hydrochloride. The small quantities recovered precluded effective purification, and the nitrogen content of the copper aspartate salts was 5 to 10 per cent less than theoretical.

Histidine—The basic amino acids, other than arginine, were precipitated with phosphotungstic acid, and this precipitate was decomposed with a butyl alcohol-ethyl ether mixture. Histidine was separated by means of its mercury salt, then regenerated with H_2S . Histidine monohydrochloride crystallized once from the 15 minute fraction but then formed an oil and refused to recrystallize. The oil contained 0.641 atom per cent N^{15} excess. Histidine flavanate was prepared from the oil; nitrogen found 12.36 per cent; theory 12.39 per cent. Histidine recovered from decomposition of the flavanate with concentrated HCl showed 0.626 atom per cent N^{15} excess. No derivatives were obtained with the 3 and 8 minute fractions, and analysis is given for the histidine fraction obtained by releasing histidine with H_2S from its mercury salt.

Lysine Fraction—No lysine or lysine picrates could be recovered from the

filtrates from the mercury salts of histidine. Samples from the entire fractions were analyzed for their N^{15} contents.

Tyrosine—Tyrosine crystallized from the filtrate of the 15 minute phosphotungstic acid precipitate. The recrystallized amino acid contained 7.55 per cent N; theory 7.74 per cent; m.p. 287° ; accepted 295° .

Monoamino Acids—Copper salts were prepared from the phosphotungstic acid (or tyrosine) filtrates, and separated on the basis of their solubility. The separated copper salts were decomposed with H_2S . Leucine crystallized from the water-insoluble copper salt fraction and the 3, 8, and 15

TABLE I
Distribution of N^{15} in Amino Acids and Amino Acid Fractions

	Atom per cent N^{15} excess			
	15 min. NH_4^+ treatment	8 min. NH_4^+ treatment	3 min. NH_4^+ treatment	90 min. N_2 treatment
Total hydrolysate	1.049	0.354	0.174	0.275
Humin	0.683	0.288	0.067	
Neuberg filtrate	0.915			0.441
"Amide" nitrogen	0.931	1.450	0.749	0.325
Arginine	0.667	0.165	0.026	0.185
Glutamic acid	2.594	0.909	0.392	0.500
Aspartic "	0.832	0.523	0.187	0.376
Histidine fraction	0.634	0.153	0.034	0.207
Lysine fraction	0.807	0.097	0.052	0.356
H_2O insoluble Cu salts	0.690	0.195	0.075	0.313
H_2O -soluble MeOH-insoluble Cu salts	0.617	0.172	0.039	0.320
" " MeOH-soluble Cu salts	0.782	0.192	0.042	0.327
Tyrosine	0.758			
Leucine	0.602	0.204	0.068	

minute leucine crystals decomposed at 292° , 292° , and 289° respectively; accepted decomposition point 295° . The leucine from the 15 minute fraction contained 10.48 per cent N; theory 10.68 per cent.

Table I summarizes the levels of N^{15} found in the various amino acids and amino acid fractions; data from earlier work with molecular N^{15} (1) are included for comparison.

DISCUSSION

The experiments described furnish a parallel with those previously reported in which *Azotobacter vinelandii* depended for nitrogen exclusively upon the fixation of molecular N_2 . In general the distribution of isotopic nitrogen is very much the same whether supplied as free nitrogen or as the ammonium ion. In comparing the two sources of nitrogen the pertinent data are how much the atom per cent excess N^{15} in a given fraction exceeds

that noted in the total hydrolysate. In general the values for humin, Neuberger filtrate, and amide nitrogen have questionable metabolic significance. It should be noted that with cultures supplied ammonium sulfate the "amide" fraction includes residual ammonia. The most noteworthy accumulation of N^{15} occurred in glutamic acid. The second highest was in aspartic acid which can be readily formed from glutamic acid by the *transaminase* in the *Azotobacter* (4). The lysine fraction occupies a rather questionable position, in some cases being relatively high in N^{15} concentration and in other cases low. The monoamino acids regularly occupy an intermediate position, and arginine and histidine are uniformly low in their N^{15} contents.

That the N^{15} content of glutamic acid exceeds that of any other compound in the cells given N^{15} in the form of the ammonium ion more strikingly than in those fixing N_2 likely results from the earlier harvest of the cells supplied the ammonium ion (3, 8, or 15 minutes compared to 90 minutes). It would be anticipated that the greater the period between the addition of an N^{15} -enriched compound and harvest of the cells the closer would be the approach toward an equilibrium in the N^{15} content of the various compounds in the cells and the less the differential in their respective concentrations.

The close similarity in the distribution of N^{15} in the *Azotobacter* supplied with the isotope as molecular nitrogen and as the ammonium ion constitutes further presumptive evidence for ammonia as an intermediate in biological nitrogen fixation (5).

SUMMARY

Actively growing cultures of *Azotobacter vinelandii* were supplied with ammonium sulfate containing N^{15} for periods of 3, 8, and 15 minutes and immediately harvested and hydrolyzed. Fractionation of the amino acids showed a distribution of N^{15} very similar to that observed when N^{15} was supplied as molecular nitrogen. Glutamic acid in each case contained the highest level of N^{15} , indicating that it occupies a key position in the nitrogen metabolism of the *Azotobacter*. All observations are compatible with the ammonia hypothesis of nitrogen fixation by this organism.

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ON THE NATURE OF TWO CARBOXYLIC ACIDS OF HIGH MOLECULAR WEIGHT OBTAINED FROM THE WAXES OF ACID-FAST BACTERIA

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Anderson and collaborators found that the volatile product obtained on heating mycolic acid (1) and bovine mycolic acid (2) under reduced pressure at a temperature of about 300° consisted of *n*-hexacosanoic acid. Anderson and Creighton (3) found that avian β -mycolic acid under similar conditions gave *n*-tetracosanoic acid. From avian α -mycolic acid they obtained, however, a pentacosanoic acid that differed from the *n* acid in melting point and crystalline appearance. A tetracosanoic acid, which differed in a similar manner from the *n* acid, was isolated by Cason and Anderson (2) from the hydrolysis products of bovine wax. Several branched chain acids of high molecular weight have been isolated from acid-fast bacteria and it was therefore suggested that the two acids just mentioned might have branched structures. The investigations of Francis, Piper, Chibnall, and their collaborators (4-9) have shown that acids and alcohols of high molecular weight obtained from natural sources, *i.e.* waxes of different origin, are very often mixtures of closely related even numbered homologues. A number of acids described in the literature have thus been found to be mixtures. The melting points of the acids obtained by Anderson *et al.* do not exclude the possibility that they might be mixtures of closely related normal chain homologues. The composition of such mixtures does not change appreciably on recrystallization and they therefore behave like single compounds. Investigations in this laboratory have shown, however, that normal chain acids and mixtures of such acids differ considerably in monolayer behavior from branched chain acids of similar molecular weight. It ought to be possible, therefore, by means of monolayer studies, which have the advantage of using only a very small amount of material, to decide whether the acids mentioned have branched structures or are mixtures of normal chain homologues.

EXPERIMENTAL

The force-area curves given by the pentacosanoic acid from avian α -mycolic acid spread on acid substrate at different temperatures are shown in Fig. 1. A thermostat-controlled Langmuir-Adam balance was used (*cf.* (10)). The low areas reached before collapse at once suggested

that the acid could not have a branched chain structure. On the other hand, at the time these experiments were carried out (in 1942) force-area curves of the type shown in Fig. 1 had not been described previously. It was subsequently found that artificial mixtures of n acids gave similar curves and they were at one time believed to be characteristic for such mixtures (11), because, owing to poor spreading, similar results were not obtained with pure n acids. In order to study these phenomena more closely it became necessary to improve the spreading technique and to construct a special recording surface balance with symmetrical compression of the film (12). It was found that proper spreading of pure acids could be effected by use of a mixture of benzene and chloroform (9:1 by volume) as solvent. The best solvents for normal long chain acids are benzene and chloroform (6). The solubility in pure benzene is too slight for the present purpose, however, and pure chloroform is also unsuitable owing to the high specific gravity of the spreading solution. If the latter has a specific gravity much higher than that of the substrate, the drops tend to fall through the surface to the bottom of the trough. The mixture just mentioned has a specific gravity of just under 1, and was found to be excellent with regard to both solvent and spreading purposes.

A detailed study, with use of the double acting balance and improved spreading technique, led to the discovery of two new surface phases in condensed monolayers of long normal chain acids. The complete phase diagram for n -docosanoic acid has been described in a previous communication (12). A large number of artificial mixtures of normal chain acids have been studied¹ and, as a general rule, it has been found that pure acids and mixtures of closely related homologues behave very similarly. If the difference in chain length between the components is large, the force-area curves deviate considerably from those given by pure acids. The curves shown in Fig. 1 for the pentacosanoic acid from avian α -mycolic acid are very similar to those given by mixtures of closely related normal homologues and are quite different from those given by the simplest branched chain acids having the same molecular weight; *i.e.*, those possessing a single methyl side chain. For comparison, the force-area curves for three pentacosanoic acids having a methyl side chain in different positions along the chain are shown in Fig. 2.

The results obtained for the tetracosanoic acid from bovine wax indicate in a similar manner that this acid also must be a mixture of normal chain homologues. At a temperature of 40° (Fig. 3) the force-area curve for this acid is nearly identical with that for n -tetracosanoic acid from avian β -mycolic acid. The difference between the synthetic n acid and the acid from avian β -mycolic acid is probably due to a small amount of

¹A detailed account of this work will be published elsewhere.

impurity in the latter (the acid gives a slight yellow discoloration with concentrated sulfuric acid at 70°).

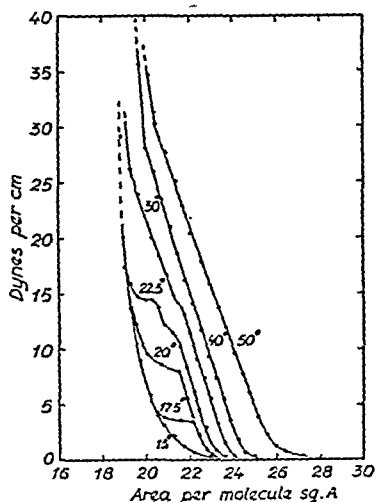


FIG. 1. Force-area curves for the pentacosanoic acid from avian α -mycolic acid spread on 0.01 N hydrochloric acid at different temperatures.

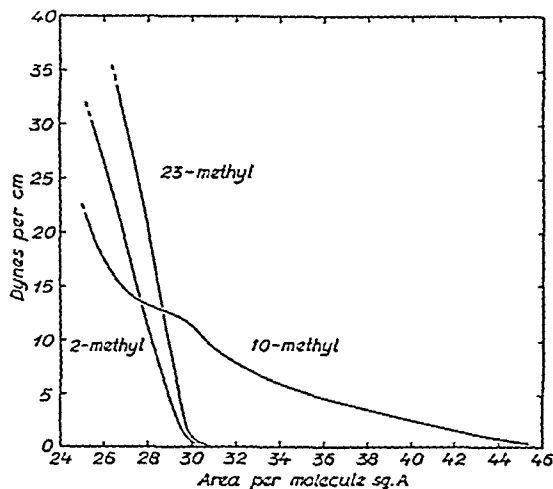


FIG. 2. Force-area curves for three pentacosanoic acids having a methyl side chain at different positions along the chain. 0.01 N hydrochloric acid substrate at 20°.

As a further check on the above results, multilayers of the acids were built from a neutral substrate containing barium ions (barium chloride 3×10^{-5} M, potassium hydrogen carbonate 4×10^{-4} M). Such multilayers contain the barium salt of the acid together with some free acid. In the case of normal chain acids the molecules of the barium salt form vertically oriented double molecules and the long x-ray spacings obtained from the multilayers correspond to twice the length of the molecules (13-15).

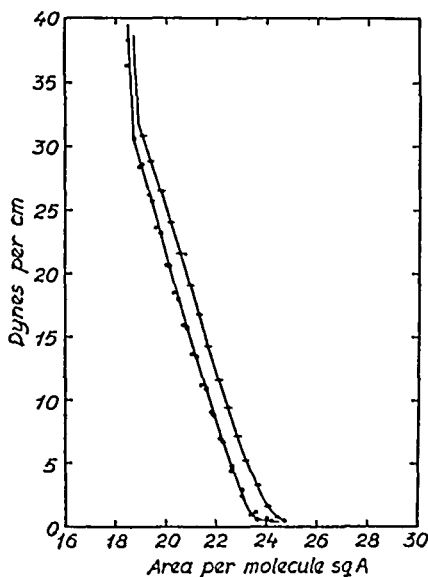


FIG. 3. Force-area curves on 0.01 N hydrochloric acid substrate at 40° for *n*-tetracosanoic acid (filled circles with bar), *n*-tetracosanoic acid from avian β -mycolic acid (open circles), and tetracosanoic acid from bovine wax (filled circles).

Mixtures of normal chain acids give spacings intermediate between those given by the components (15). Multilayers of branched chain acids, in the few cases that have been studied so far, give x-ray spacings which are shorter than the length of the double molecule but longer than that of a single molecule, indicating tilted double molecules (16). Multilayers of the pentacosanoic acid from avian α -mycolic acid and the tetracosanoic acid from bovine wax gave long x-ray spacings of 67.5 and 65.2 Å respectively. Multilayers of *n*-pentacosanoic acid and *n*-tetracosanoic acid² built under identical conditions gave long spacings of 67.7 and 65.3 Å respectively. These results are obviously consistent with the view that the two natural acids are mixtures of closely related normal chain homologues.

²The *n* acids used here were original synthetic specimens obtained from Professor Francis of Bristol (cf. (17)).

Some attempts have been made to settle the composition of the pentacosanoic acid from avian α -mycolic acid. For this acid Anderson and Creighton (3) report a molecular weight (by titration) of 379.8 (379.6, 380). As *n*-tetracosanoic and *n*-hexacosanoic acids have been obtained by pyrolysis of other mycolic acids, it is reasonable to suggest that the pentacosanoic acid might be a mixture of these two acids. If so, the molecular weight indicates a mixture of 60 mole per cent *n*-C₂₄ and 40 per cent *n*-C₂₅. From the melting point curve for the binary mixture of these two acids constructed by Piper, Chibnall, and Williams (6) the melting point of such a mixture would be about the same, *i.e.* 78–79°, as that reported (3) for the avian pentacosanoic acid. The monolayer results of the artificial mixture, however, differ from those of the natural compound in that the temperatures at which the various surface phase transitions take place are higher for the artificial mixture. It seems necessary, therefore, to assume the presence of a third component in the natural product. A minute amount of unsaturated material is indicated by the fact that a slight color is produced when the specimen is treated with concentrated sulfuric acid at a temperature of 70°.

We are greatly indebted to Professor R. J. Anderson for specimens of the natural products and for his interest in the work. The expenses involved in this work were defrayed by grants from the National Swedish Antituberculosis Association and the Rockefeller Foundation. We are indebted to Mrs. K. Nilsson for assistance in the experimental work.

SUMMARY

A mono- and multilayer study of a pentacosanoic acid obtained by pyrolysis of avian α -mycolic acid and a tetracosanoic acid derived from the wax of the bovine tubercle bacillus indicates that both these acids are mixtures of closely related normal chain homologues.

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MECHANISM OF INHIBITION OF PHOSPHATASE ACTIVITY BY GLYCINE

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Glycine and other α -amino acids in *very low concentrations* have been shown to increase the apparent activity of several enzymes: urease, the various amylases, pancreatic lipase, tyrosinase, yeast peptidase, and the phosphatases (1-3). Occasional observations in the literature indicate, however, that higher concentrations of α -amino acids exert an inhibiting effect on enzyme action (1, 4-7). Thus Kato's study of the effect of glycine on urease activity contains data showing that glycine inhibits this enzyme at concentrations greater than about 0.04 M (4). Bodansky observed that tissue phosphatase activity was retarded by glycine and other α -amino acids in concentrations greater than about 0.01 to 0.001 M, depending upon the particular α -amino acid employed (1).

In view of these instances of enzyme inhibition by α -amino acids, it was considered that the nature of the effect warranted more complete investigation. The present paper is concerned with the mechanism of inhibition of bone and intestinal phosphatase preparations by glycine.

EXPERIMENTAL

The methods for preparing the phosphatase extracts and for measuring their activity have been previously described (1, 8). Dialyzed phosphatase extracts of rat bone, rat intestine, human intestine, and cat bone were employed. The concentration of the substrate used, sodium β -glycerophosphate, depended upon the particular experiment. The concentration of the buffer, sodium diethyl barbiturate, was 0.5 gm. per 100 cc. of hydrolysis mixture. All reaction velocity determinations were carried out at optimal pH (9.0 to 9.2), attained by incorporating the necessary amount of acid or alkali in the buffered mixture. Since it had been previously shown that concentrations of about 0.01 to 0.001 M magnesium ion and 0.006 M glycine are necessary for optimal activity of phosphatase and for direct proportionality between such activity and enzyme concentration (1), the inhibitory effects of glycine were determined in mixtures containing these concentrations of magnesium ion and glycine. Thus, any concentration of glycine noted in the text represents that present in addition to 0.006 M glycine. The sources of the compounds were as follows: glycine (Eastman

Kodak), monomethylglycine (Hoffmann-La Roche), glycine ethyl ester (Hoffmann-La Roche). Dimethylglycine was prepared according to the method of Michaelis and Schubert (9).

The reaction velocity was determined as previously described (8), usually in duplicate hydrolyses. The amount of phosphorus liberated as inorganic phosphate per cc. of hydrolysis mixture was determined at

TABLE I

Inhibition of Bone Phosphatase Activity by 0.0625 M Glycine at Varying Substrate (Sodium β -Glycerophosphate) Concentrations

Concentration of rat bone phosphatase, Preparation RBM-d, 12.5 per cent by volume in hydrolysis mixture. Theoretical reaction velocities in absence and presence of glycine calculated in accordance with Equations 1 and 2 (see the text), respectively. $K_s = 0.0019$ mole per liter; $K'_s = 0.0019$ mole per liter.

Concentration of Na β -glycerophosphate	Phosphorus liberated as phosphate per cc. per min.			
	No inhibition		0.0625 M glycine	
	Observed	Calculated	Observed	Calculated
M	γ	γ	γ	γ
0.00127	0.88	0.80	0.35	0.33
0.00134	0.87	0.82		
0.00191	0.99	1.00		
0.00319	1.11	1.25	0.53	0.50
0.00635	1.37	1.54	0.65	0.64
0.0127	1.81	1.74	0.72	0.73
0.0254	1.89	1.84	0.77	0.78
∞	3.12*	3.12	0.83*	0.83

* Extrapolated.

three time intervals, spaced as equally apart as possible, during the zero order portion of the reaction. All hydrolyses were conducted at 25° in a water thermostat regulated to within 0.01–0.05°.

Results

Inhibition of Bone Phosphatase—Table I shows the effect of 0.0625 M glycine on the velocities of action of rat bone phosphatase, Preparation RBM-d, as the concentration of substrate, sodium β -glycerophosphate, was varied. The reciprocals of the reaction velocities in the absence of glycine were plotted against the reciprocals of the substrate concentrations, in accordance with the Lineweaver-Burk transposition of the Michaelis-Menten expression (10, 11),

$$\frac{1}{V_0} = \frac{K_s}{V_{\max.}} \cdot \frac{1}{S} + \frac{1}{V_{\max.}} \quad (1)$$

where V_0 is the reaction velocity in the absence of inhibitor at concentration S of the substrate, V_{\max} is the reaction velocity in the absence of inhibitor at maximal (theoretically infinite) concentration of substrate, and K_s is the dissociation constant of the assumed, intermediate enzyme-substrate complex. A straight line was drawn through the experimental points and the following values were obtained from the plot: $K_s = 0.0019$ mole per liter; $V_{\max} = 2.00 \gamma$ of P liberated per cc. per minute.

A similar plot was made of the reaction velocities in the presence of 0.0625 M glycine in accordance with the following Lineweaver-Burk expression.

$$\frac{1}{V} = \frac{1}{V_{\max}} \left(K_s + \frac{K_s I}{K_I} \right) \frac{1}{S} + \frac{1}{V_{\max}} \quad (2)$$

V is the reaction velocity in the presence of concentration of inhibitor, I , at substrate concentration; $S \cdot V_{\max}$ is the reaction velocity at maximal substrate concentration in the presence of inhibitor. K_I is the dissociation constant of the assumed intermediate enzyme-inhibitor complex and $K'_s = K_s + (K_s I / K_I)$ is the dissociation constant of the intermediate enzyme-substrate complex in the presence of inhibitor. A straight line drawn through the experimental values gave the following: $V_{\max} = 0.83 \gamma$ of P liberated per cc. per minute; $K'_s = 0.0019$ mole per liter. The third and fifth columns of Table I show the values for the reaction velocities calculated by using these values for V_{\max} in the presence and absence of glycine and for K_s and K'_s . Except for the reaction velocities at 0.00319 and 0.00635 M substrate in the absence of glycine, these calculated values are in very good agreement with the experimentally determined reaction velocities. The findings that V_{\max} in the presence of glycine was less than V_{\max} without inhibitor and that K'_s had, within experimental error, the same value as K_s , show that the inhibition of rat bone phosphatase by glycine was wholly non-competitive.

In order to obtain the value for the dissociation constant of the assumed bone phosphatase-glycine complex, the velocities of action were determined at varying glycine concentrations and constant substrate concentration, 0.0127 M sodium β -glycerophosphate (Table II). The values for K'_I , the dissociation constant of an enzyme-inhibitor complex formed non-competitively, were calculated in accordance with the following transposition of the Michaelis-Menten expression (10),

$$K'_I = \frac{I}{\frac{V_0}{V} - 1} \quad (3)$$

V_0 is the velocity without inhibitor and V the velocity at the concentration, I , of the inhibitor. It may be seen from Table II that the values of K'_I ,

were the same, within experimental error, when the concentration of glycine was varied from 0.00625 to 0.250 M. The average value of K'_I is 0.039 (s.d. = 0.0045). Since Equation 3 does not contain any terms involving substrate concentration, the value for K'_I is the same at all substrate concentrations.

Inhibition of Intestinal Phosphatase—Table III, Column 2, shows the velocities of action of the rat intestinal phosphatase, Preparation RIK-d, as the concentration of substrate was varied. The reciprocals of these

TABLE II

Non-Competitive Nature of Inhibition of Rat Bone Phosphatase by Glycine

Concentration of rat bone phosphatase, Preparation RBM-d, 12.5 per cent by volume in hydrolysis mixture. Concentration of sodium β -glycerophosphate, 0.0127 M. Dissociation constant, K'_I , calculated in accordance with Equation 3 (see the text). $K'_I = (I/(V_0/V) - 1)$.

Concentration of glycine	P liberated as phosphate per cc. per min.	$\frac{V_0}{V} = \frac{\text{velocity without glycine}}{\text{velocity with glycine}}$	K'_I
M	γ		mole per l.
0.0	1.81		
0.00625	1.52	1.19	0.033
0.0125	1.42	1.28	0.045
0.0313	0.93	1.95	0.033
0.0625	0.72	2.52	0.041
0.125	0.43	4.22	0.039
0.250	0.26	6.97	0.042

velocities in the absence of glycine were plotted against the reciprocals of the corresponding concentrations of substrate and a straight line drawn through the experimental points in accordance with Equation 1. The following values were obtained: $K_s = 0.0029$ mole per liter, $V_{\max.} = 3.12 \gamma$ of P liberated per cc. per minute. Column 3 shows that the reaction velocities calculated on the basis of these values are in good agreement with the experimentally determined velocities. Column 4 shows the reaction velocities in the presence of 0.0625 M glycine. The reciprocals of these velocities were plotted against the reciprocals of the substrate concentrations and a straight line was drawn through the resulting points in accordance with Equation 2. According to this plot, $V_{\max.}$ in the presence of glycine was 1.42 γ of P liberated per cc. per minute, and $K'_I = K_s + (K_s I / K_I)$ was 0.0062 mole per liter. That in this case also the experimental points fell very close to the straight line drawn through them is attested by the excellence of the agreement between the calculated (Column 5) and the experimentally determined velocities (Column 4).

The finding that $V_{\max.}$ with glycine was lower than $V_{\max.}$ without glycine indicated the non-competitive nature of the inhibition by glycine. On the

other hand, the finding that $K' > K$, showed that there was also a competitive component in this inhibition. That enzyme inhibitors need not be either wholly competitive or wholly non-competitive has, of course, been previously noted in a number of instances (12).

The relative magnitude of the competitive and non-competitive components of the inhibition of rat intestinal phosphatase by glycine was determined by the following calculations and experiments. At maximal, i.e. theoretically infinite, substrate concentration, whatever inhibition is

TABLE III

Inhibition of Intestinal Phosphatase Activity by 0.0625 M Glycine at Varying Substrate (Sodium β -Glycerophosphate) Concentrations

Concentration of rat intestinal phosphatase, Preparation RIK-d, 12.5 per cent by volume in hydrolysis mixture. Theoretical reaction velocities in absence and presence of glycine calculated in accordance with Equations 1 and 2 (see the text), respectively. $K_s = 0.0029$; $K'_s = 0.0062$ mole per liter.

Concentration of Na β -glycero- phosphate	Phosphorus liberated as phosphate per cc. per min.					Ratio of non- competitive to total inhibition*
	No inhibition		0.0625 μ glycine		If inhibition were solely non- competitive* (6)	
	Observed (2)	Calculated (3)	Observed (4)	Calculated (5)		
(1)	(2)	(3)	(4)	(5)	(6)	(7)
μ	γ	γ	γ	γ	γ	<i>per cent</i>
0.00127	0.96	0.94	0.24	0.24	0.43	74
0.00254	1.39	1.45	0.41	0.41	0.63	78
0.00635	2.09	2.13	0.71	0.72	0.94	83
0.0127	2.47	2.55	0.95	0.95	1.13	88
0.0254	2.97	2.81	1.17	1.13	1.34	91
0.0508	2.89	2.96				
∞	3.12†	3.12	1.42†	1.42	1.42	100

* See the text for the method of calculating these values.

† Extrapolated.

present is due entirely to the non-competitive component (11, 12). As may be calculated from Table III, the ratio V/V_0 at this concentration was 0.45. The reaction velocities at the other substrate concentrations in the absence of glycine were multiplied by this factor to give the velocities that would result if the inhibition by glycine were solely non-competitive (Table III, Column 6). These velocities were then compared with those actually obtained in the presence of 0.0625 M glycine. The inhibitions were then calculated, and the non-competitive component expressed as a fraction of the total inhibition. For example, at 0.00127 M substrate, the total inhibition amounted to $0.96 - 0.24$ or 0.72γ of P per cc. per minute; the calculated inhibition that would result if it were solely non-competitive would be $0.96 - 0.43$ or 0.53γ of P per cc. per minute. Hence, the non-competitive component was 74 per cent in this instance. As may be seen

from Table III, this component constituted from 74 to 91 per cent of the total inhibition at various substrate concentrations, being greater at the higher substrate concentrations.

Table IV shows the inhibition of intestinal phosphatase activity by various concentrations of glycine at a substrate concentration of 0.0127 M. In the third column, these velocities are calculated as fractions of the velocities without glycine ($V:V_0$). Application of Equation 3, the expression for non-competitive inhibition, to the reaction velocities at maximal substrate concentration in the presence and absence of 0.0625 M

TABLE IV

Inhibition of Intestinal Phosphatase Activity at Varying Concentrations of Glycine

Concentration of rat intestinal phosphatase, Preparation RIK-d, 12.5 per cent by volume in hydrolysis mixture. Concentration of sodium β -glycerophosphate, 0.0127 M.

Concentration of glycine	Phosphorus liberated as phosphate per cc. per min.				Ratio of non-competitive to total inhibition*
	Observed		If inhibition were solely non-competitive*		
		Fraction of uninhibited velocity		Fraction of uninhibited velocity	
<i>M</i>	<i>γ</i>	<i>per cent</i>	<i>γ</i>	<i>per cent</i>	<i>per cent</i>
0.0	2.47	100	2.47	100	
0.0125	1.84	75	1.98	80	80
0.0313	1.53	62	1.54	63	97
0.0625	0.95	39	1.11	45	90
0.125	0.64	26	0.73	29	96
0.188	0.50	20	0.52	22	98
0.250	0.36	15	0.42	17	98

* See the text for the method of calculating these values.

glycine yielded a value of 0.051 mole per liter for K' , the dissociation constant. By substituting this value and the velocity, 2.47 γ of P per cc. per minute, at 0.0127 M substrate concentration in the absence of glycine (V_0) in Equation 3, it was possible to obtain, within the experimental errors involved, the reaction velocities that would exist at the various glycine concentrations *if the inhibition were solely non-competitive*. The ratios of non-competitive to total inhibition were then calculated (last column, Table IV). For example, at 0.250 M glycine, the total inhibition was $2.47 - 0.36$ or 2.11 γ of P per cc. per minute; the inhibition that would exist if it were solely non-competitive would be $2.47 - 0.42$ or 2.05 γ of P per cc. per minute. Hence the non-competitive component in this instance was 98 per cent of the total. It may be seen that, at a concentration of 0.0127 M substrate, the non-competitive inhibition constituted

TABLE V

Inhibition of Phosphatase Activity by Glycine and Glycine-Substituted Compounds

Concentration of phosphatase, 12.5 per cent by volume in hydrolysis mixture.
 Concentration of sodium β -glycerophosphate, 0.0127 M.

Phosphatase preparation	Concentration of inhibitor	Phosphorus liberated as phosphate per cc. per min.			
		Glycine	Glycine ethyl ester	Monomethylglycine	Dimethylglycine
	M	γ	γ	γ	γ
CaBA-d, cat bone phosphatase	0.0	1.05	1.05	1.05	0.97
	0.0125	0.83	0.78		
	0.025	0.63	0.65		0.91
	0.028			1.04	
	0.0375	0.47	0.64		
	0.0625	0.35			0.94
	0.096			0.82	
	0.125		0.36		1.01
	0.425	0.06			
	0.50				0.75
MID-d, human intestinal phosphatase	0.0	1.13	1.13		1.13
	0.00625	1.04	1.01		
	0.0125	0.96	0.96		
	0.0250	0.71	0.85		1.05
	0.050	0.50	0.75		
	0.0625				0.99
	0.125		0.44		0.92
	0.156	0.24			

TABLE VI

Concentrations of Glycine and Substituted Glycine Compounds Required to Produce 50 Per Cent Inhibition of Phosphatase Activity

These concentrations have been estimated from the experimental values recorded in Table V and in accordance with the method described in the text.

Compound	Concentration required to produce 50 per cent inhibition	
	Bone phosphatase, Preparation CaBA-d	Intestinal phosphatase, Preparation MID-d
	M	M
Glycine	0.030	0.043
" ethyl ester	0.059	0.032
Monomethylglycine	0.37	
Dimethylglycine	1.6	1.4

80 to 98 per cent of the total inhibition, being higher at the higher concentrations of glycine.

Inhibition of Phosphatase Activity by Substituted Glycine Compounds—In Table V are shown the inhibitions of cat bone phosphatase, Preparation CaBA-d, and human intestinal phosphatase, Preparation MID-d, by glycine, glycine ethyl ester, monomethylglycine, and dimethylglycine. The reciprocals of the reaction velocities were plotted against the concentrations of these various inhibitors; from the straight lines drawn through the experimental points, the concentrations at which 50 per cent inhibition occurred were estimated. Table VI shows that about twice as high a concentration of glycine ethyl ester as of glycine was necessary to produce the same degree of inhibition. The introduction of methyl groups into the amino group led to a much more marked decrease in the inhibition. Thus, about 10 times as high a concentration of monomethylglycine and about 50 times as high a concentration of dimethylglycine as of glycine were necessary to produce 50 per cent inhibition of cat bone and human intestinal phosphatase activity.

DISCUSSION

Michaelis and Menten (10) assumed that enzyme inhibition was due to the formation of an inactive but dissociable enzyme-inhibitor complex. A considerable number of studies have substantiated this assumption (10-14). Michaelis and Menten (10) also pointed out that the inhibitor may combine with the enzyme either so as to decrease its active mass without displacing it from its combination with the substrate (non-competitive inhibition) or so as to compete with the substrate (competitive inhibition). In the present study it has been shown that the inhibition of rat bone phosphatase by glycine is wholly non-competitive and in good agreement with the mathematical formulations for such inhibition. The dissociation constant of the rat bone phosphatase-glycine complex was found to be 0.039 (s.d. = 0.0045) mole per liter.

In contrast, the inhibition of rat intestinal phosphatase by glycine is mixed, being both competitive and non-competitive, but largely the latter. At a concentration of 0.0625 M glycine, the non-competitive component ranged from 74 per cent of the total inhibition at 0.00127 M substrate to 91 per cent at 0.0254 M substrate. This relative increase in the magnitude of the non-competitive component is to be expected, since competitive inhibition decreases with increasing substrate concentration.

The formation of the phosphatase-glycine complex appears to involve both the carboxyl and the amino groups of glycine. Esterification of glycine reduced the inhibition considerably, and substitution of two methyl groups for the hydrogen atoms in the amino radical practically nullified the inhibition. The extension of the studies reported in the present work to bone and intestinal phosphatases of other species, to other tissue phos-

phatases, and to α -amino acids other than glycine naturally suggests itself. It would also be of interest to elaborate the present observations on inhibition so as to determine whether blocking the carboxyl and amino groups may alter not only the degree, but also the type of inhibition.

SUMMARY

1. The Michaelis-Menten dissociation constant of the intermediate enzyme-substrate complex has been found to be 0.0019 mole per liter for the action of rat bone phosphatase on sodium β -glycerophosphate, and 0.0029 mole per liter for that of rat intestinal phosphatase on this substrate.

2. Glycine in concentrations higher than the 0.006 M necessary for optimal activity inhibits the action of phosphatase. The glycine inhibition of rat bone phosphatase is entirely non-competitive in type; the dissociation constant of the assumed rat bone phosphatase-glycine complex is 0.039 mole per liter. The glycine inhibition of rat intestinal phosphatase is mixed, being both competitive and non-competitive. Under the conditions of variation in the concentrations of substrate and glycine here investigated, the non-competitive component of the inhibition ranged from 74 to 98 per cent of the total inhibition.

3. The inhibition of phosphatase action by glycine depends to a very considerable degree upon the intactness of the carboxyl and amino groups of glycine. Esterification of the carboxyl group reduced the inhibition to about one-half its value. Introduction of methyl groups into the amino radical decreased the inhibition much more markedly.

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THE DISTRIBUTION OF CYTOCHROME OXIDASE AND SUCCINOXIDASE IN THE CYTOPLASM OF THE MAMMALIAN LIVER CELL

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The relation between enzymatic functions and cell structure remains one of the most interesting and least explored problems confronting the cytologist and biochemist. Investigations by various methods have yielded a certain amount of information on the subject, but the validity of the results, especially those secured by microscopical stain reactions, has been questioned. The present day knowledge regarding the distribution of enzymes in the cell is mostly derived from histochemical studies such as those of Gierke, Graeff, and others (1-4), from investigation by the ingenious microtechniques of Linderström-Lang and Holter (5), and from the more recent studies on the analysis of isolated parts of cells (6-11).

The experiments described in the present report offer additional data on the distribution of two enzyme systems, cytochrome oxidase and succinoxidase, in the cytoplasm of the liver cell of the rat. In these experiments, use was made of the observation of Claude (12, 13) that an extract suitable for fractionation by differential centrifugation can be prepared from animal tissues in such a way that it consists almost entirely of material from the cytoplasm, the nuclei being removed practically uninjured, together with residual intact cells, by preliminary centrifugation. This "cytoplasmic extract" can be fractionated upon further centrifugation into the following three distinct portions: (1) large granules, 0.5 to 2 μ in diameter, separated by 25 minutes centrifugation at $2000 \times g$, and representing mitochondria for the most part; (2) small particles or microsomes, 60 to 150 $m\mu$ in diameter, separated by 90 minutes centrifugation at $18,000 \times g$; and (3) the supernatant fluid remaining after the two sedimentable fractions have been removed.

The three fractions just described were examined for their cytochrome oxidase and succinoxidase content. In the present experiments, as in additional ones of the same type that involve other enzymes and will be reported in subsequent papers, attention was paid not only to the specific activity of the respective preparations in terms of their Q values but also to another quantitative aspect of the results by determining the total amount of activity recovered with each fraction, and expressing it in percentage of the total activity possessed by the original extract.

Experiments are also under way in which a comparison is being made of the activity of the various cell fractions derived from both normal liver and liver of animals treated with the carcinogen, *p*-dimethylaminoazobenzene, in order to follow, if possible, the changes brought about by the chemical in the course of the malignant transformation of the hepatic cells. The preliminary results obtained in the study are included in this report.

EXPERIMENTAL

Materials and Methods

Animals and Diet—The animals used in most of the experiments were young adult albino rats of the Wistar strain, fed a diet of bread and milk, and weighing 125 to 150 gm. In four experiments, the rats were of the Sherman strain and were fed either a basic diet (control group) consisting of unpolished rice (Texas brand), washed casein (Merck), olive oil, and carrot (14), or the same basic diet, to which *p*-dimethylaminoazobenzene was added to a final concentration of 0.06 per cent (test group).

Preparation and Fractionation of Liver Extract—The procedure by which the liver extract was prepared and subsequently fractionated in the centrifuge has been described in detail in preceding papers (12, 13). In brief, the method was as follows:

From ten to fifteen rats were used in a single experiment. Each animal was killed by a blow on the head and allowed to bleed profusely through a neck incision; its liver was removed without delay and placed on ice. The pooled livers were forced in small fragments through a masher fitted with a 1 mm. mesh screen that retained the connective tissue framework and allowed the parenchymatous part of the organs to be collected as a pulp. The liver pulp was ground alone in a mortar for about 3 minutes; the solvent, 0.85 per cent NaCl solution, was added very slowly at first, then more rapidly until a final volume equivalent to 5 times the weight of the pulp had been used. The resulting suspension was submitted to three successive centrifugations of 3 minutes at $1500 \times g$, and the sediments, composed of tissue debris, unbroken cells, free nuclei, and red corpuscles, were discarded. The supernatant solution from the last centrifugation will be referred to as the liver extract.

The liver extract, which contained for the most part elements derived from the cytoplasm of the hepatic cells (12, 13), was subjected to fractionation by means of differential centrifugation, its constituents being segregated into the three main portions already mentioned, according to the scheme illustrated diagrammatically in Fig. 1. In this fractionation a single centrifuge type was used, namely the SB, size 1 model, manufactured by the International Equipment Company, Boston. The preliminary centrifugation at $1500 \times g$ was accomplished by means of the horizontal yoke

No. 242 with Pyrex cups of 250 ml. capacity; separation and washing of the large granules were carried out under a force of $2000 \times g$ in the conical head No. 823, with narrow neck Pyrex tubes of 50 ml. capacity; the microsomes were separated by means of the multispeed attachment and No. 295 head.

The initial separation, or concentration, of the large granules was accomplished by submitting the liver extract to 25 minutes centrifugation at

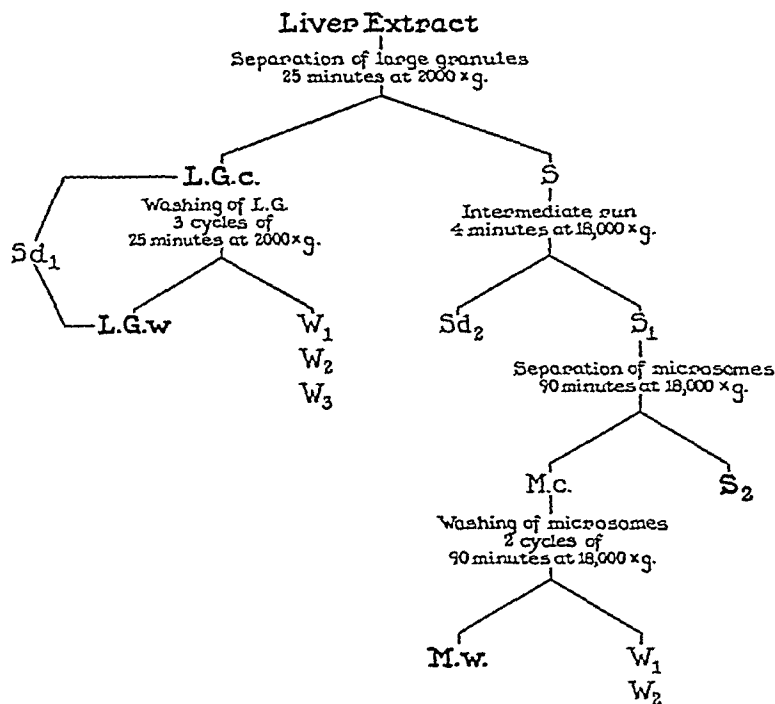


FIG. 1. Fractionation of liver extract by differential centrifugation: diagram of procedure. The fractions especially investigated were L.G.c., L.G.w., S₂, and M.w. (see the text).

$2000 \times g$. The supernatant (S) was retained for further fractionation. The main portion of the sediment was resuspended in a known volume of 0.85 per cent NaCl solution and will be referred to as the concentrated large granule preparation (L.G.c.). There remained a small bottom layer in the pellet that contained tissue debris. This fraction (Sd₁) was reserved separately.

In order to effect purification and to free the preparation from the soluble substances of the original liver extract, the large granules were washed by

submitting them to three successive cycles of centrifugation at $2000 \times g$ for 25 minutes with intervening resuspensions. The opaque, yellowish sediment from the last centrifugation was resuspended in saline and will be referred to as the washed large granule preparation (L.G.w.). At each successive centrifugation, a diminishing fraction at the bottom of the pellet, which consisted of a few residual red cells, nuclei, and agglutinated large granules, was removed and added to fraction Sd_1 .

The supernatant (S), set aside when the mass of the large granules was removed, still contained large granules in small amount. In order to insure greater homogeneity for the microsome fraction the remaining large granules were discarded, together with a portion of the microsome substance itself, by a centrifugation of 5 minutes at $18,000 \times g$. The supernatant S_1 was saved, while the resulting mixed sediment (Sd_2) was resuspended in saline and used for determination of solids and activity tests.

Separation of the microsome material was then brought about by submitting S_1 to centrifugation at $18,000 \times g$ for 90 minutes. The supernatant S_2 was saved and constituted the third liver fraction. The sediment from this high speed centrifugation appeared as a pellet of jelly-like material, completely transparent and dark amber in color. The entire deposit was resuspended in saline to give an opalescent suspension which will be referred to as the concentrated microsome fraction (M.c.).

Washing of the microsome material was accomplished by submitting it to two cycles of centrifugation at $18,000 \times g$ for 90 minutes and resuspension in saline. The deposit from the last centrifugation will be referred to as the washed microsome preparation (M.w.).

Determination of Cytochrome Oxidase Activity—The determination of cytochrome oxidase was made aerobically at 38° according to the method of Schneider and Potter (15). The Warburg manometric vessel contained 0.35 ml. of enzyme preparation, 0.1 ml. of 0.5 M phosphate buffer, pH 7.4, 1.0 ml. of 1.3×10^{-4} M cytochrome *c* in 0.85 per cent NaCl solution, and 0.15 ml. of 0.005 M $AlCl_3$. The side arm contained 0.15 ml. of 0.114 M sodium ascorbate, the center well 0.2 ml. of 5 N KOH. A control vessel, containing all the reagents except the enzyme preparation, was run in parallel. The ascorbate solution was tipped into the vessel after equilibration for 15 minutes. 2 to 3 minutes were then allowed for reestablishment of temperature equilibrium, and the manometer readings were made thereafter every 5 or 10 minutes. The average volume of oxygen absorbed during the first 20 minute period was used in calculating the Q_0 , values for each preparation (microliters of oxygen taken up per hour per mg. of dry weight). In addition, the total activity of each fraction was calculated from the aliquot used in the flask and expressed in per cent of the total activity exhibited by the unfractionated extract (recovery values).

It was noted, as pointed out elsewhere (16), that the autoxidation of ascorbic acid varied somewhat from one experiment to the next and was usually depressed in the presence of active cytochrome oxidase preparations. For this reason, it was necessary to make the determination at three levels of enzyme activity for each fraction and to calculate the ascorbic acid autoxidation by extrapolation to zero enzyme concentration. This procedure was accepted as valid, since it yielded a linear relation between oxygen uptake and amount of enzyme preparation. Use was made of a 20 minute period for calculation of Q_{O_2} and recovery values, since the oxygen uptake was regularly constant during that interval but tended, in some instances, to decrease slowly at later periods. The endogenous oxygen uptake, without substrate, of all preparations at the dilutions used for the cytochrome oxidase determination was found in preliminary experiments to be negligible. A second control flask to correct for endogenous oxygen uptake was therefore not used.

Determination of Succinoxidase Activity—The determination of aerobic succinoxidase activity was carried out manometrically at 38° (15). The vessel contained 0.80 ml. of enzyme preparation; 0.1 ml. of 0.5 M phosphate buffer, pH 7.4; 0.4 ml. of cytochrome *c* solution (1.3×10^{-4} M); 0.15 ml. of 0.005 M $CaCl_2$; and 0.15 ml. of 0.005 M $AlCl_3$. The side arm contained 0.15 ml. of 0.5 M sodium succinate, and 0.2 ml. of 5 N KOH was placed in the center well. A control without substrate for determination of endogenous oxygen uptake was necessary only in the case of the extract, the endogenous uptake of this preparation being approximately 10 per cent of that occurring in the presence of succinate. The assay of each preparation was made at two or more levels of enzyme activity, and the procedure followed in equilibrating the vessels, reading the manometers, and calculating the Q_{O_2} and per cent recovery of activity from the extract was similar to that described for the cytochrome oxidase determination. The oxygen uptake observed with succinate as substrate was linearly proportional to the amount of enzyme preparation at the dilutions used in the present determinations.

Determination of Anaerobic Succinic Dehydrogenase Activity—The demonstration that ferricyanide is reduced in the presence of tissue slices and succinate with the formation of acid and that the rate of acid formation can be measured anaerobically in bicarbonate medium (17) has provided a convenient method for the determination of succinic dehydrogenase. The results of preliminary experiments indicated not only that the ferricyanide method was applicable to cell-free tissue suspensions as well as to tissue slices, but also that the rate of CO_2 production approached fairly closely the theoretical value predicted from the rate of oxygen uptake in aerobic determinations (*i.e.*, 4 microliters of CO_2 evolved anaerobically per 1 microliter of O_2 absorbed aerobically).

The determination of succinic dehydrogenase by this method was made as follows: 1.5 ml. of 0.05 M NaHCO_3 , 0.2 ml. of 0.5 M sodium succinate, 0.8 ml. of 0.85 per cent NaCl, and 0.5 ml. of the enzyme preparation were mixed in a Warburg vessel, and 0.4 ml. of an 8 per cent solution of $\text{Na}_2\text{Fe}(\text{CN})_6$ in 0.025 M NaHCO_3 was placed in the side arm. The vessel was quickly gassed with a 5 per cent CO_2 -95 per cent N_2 mixture and equilibrated at 38° for 15 minutes. The contents of the side arm were then added to the vessel, 2 minutes were allowed for reestablishment of temperature equilibrium, and readings were taken thereafter at 5 minute intervals. A control vessel without succinate was run in parallel. $Q_{\text{CO}_2}^{\text{N}_2}$ values were calculated from the average CO_2 output observed during the initial 10 minute period. The evolution of CO_2 was constant over this period but at later intervals declined at a fairly rapid rate (approximately 10 per cent per 5 minutes).

Results

Distribution of Cytochrome Oxidase and Succinoxidase in Cytoplasmic Extract of Normal Rat Liver—In three consecutive experiments the distribution of cytochrome oxidase and succinoxidase among the three main fractions of normal rat liver was determined. The results are shown in Table I. It can be seen that the removal from the extract of all particulate components sedimentable at $18,000 \times g$ for 90 minutes ($\text{E} \rightarrow \text{S}_2$) resulted in complete removal of demonstrable cytochrome oxidase and succinoxidase activity. An average of 70 per cent of the former and 74 per cent of the latter enzyme system was recovered in the unwashed mitochondrial fraction (L.G.c.). Only a small proportion of the activity (less than 4 per cent of the cytochrome oxidase and approximately 7 per cent of the succinoxidase) was found in the microsome fraction (M.w.).

Several interesting points are brought to light when the Q_{O} and recovery values for the unwashed mitochondrial fraction (L.G.c.) are compared with corresponding figures observed after the granules had been washed three times with saline (L.G.w.). The cytochrome oxidase Q value rose sharply on washing and almost all of the cytochrome oxidase originally present in the unwashed granules remained associated with these elements. The succinoxidase Q value, however, rose only slightly upon washing, and the per cent recovery values indicated that an appreciable proportion of the total succinoxidase activity had been lost. It was evident, therefore, that the washing procedure resulted in definite purification of cytochrome oxidase with very little loss of enzyme activity, whereas purification of succinoxidase was only slight and accompanied by a considerable loss of the total enzyme activity.

Succinic Dehydrogenase Activity of Particulate Fractions—Since the uptake of oxygen by the succinoxidase system is effected through the mediation of

cytochrome oxidase, it is conceivable that the latter enzyme may, under certain conditions, become the limiting factor in the aerobic oxidation of succinate. This possibility was remote in the case of the mitochondrial fraction which showed high cytochrome oxidase activity but could not be eliminated, on the basis of the data in Table I, in the case of the microsomes which contained very little cytochrome oxidase.

In order to obtain a more direct measure of activity against succinate, both sedimentable components of the liver extract were tested anaerobically

TABLE I
Distribution of Cytochrome Oxidase and Succinoxidase in Cytoplasmic Extract of Rat Liver

Liver fraction	Experiment No.	Dry weight of preparation	Total volume of preparation	Cytochrome oxidase		Succinoxidase	
				Q _{O₂}	Per cent recovery	Q _{O₂}	Per cent recovery
		mg. per ml.	ml.				
E (liver extract)	1	26.0	250	27	(100)	8.4	(100)
	2	29.8	250	32	(100)	9.0	(100)
	3	24.7	315	36	(100)	10.0	(100)
L.G.c. (large granules, unwashed)	1	32.0	20.0	202	74	66	78
	2	44.0	20.0	185	68	58	76
	3	38.8	25.0	195	67	55	69
L.G.w. (large granules, washed 3 times)	1	20.2	20.0	318	73	63	47
	2	28.2	20.0	272	64	70	59
	3	25.2	25.0	295	66	62	50
M.w. (microsomes, washed 2 times)	1	27.5	22.5	<10	<4	7	8
	2	38.6	22.3	<10	<4	4	5
	3	23.5	33.8				
S ₂ (supernatant after removal of large granules and microsomes)	1	16.6		0	0	0	0
	2	17.9		0	0	0	0
	3	16.6					

for succinic dehydrogenase content by the ferricyanide method. The results of these determinations, which are summarized in Table II, demonstrate that the ratio, activity of large granules to activity of microsomes, in the oxidation of succinate, was roughly the same whether measured aerobically or anaerobically. The results thus indicate that cytochrome oxidase was not a limiting factor in the determinations of the aerobic succinoxidase activity of the microsomes.

Additional Studies of Succinoxidase System of Mitochondrial Fraction—As shown by the recovery values in Table I, repeated washing of the large granule preparations in isotonic saline solution resulted in a considerable loss in their total succinoxidase content. Although this effect is not entirely

understood, it is probably related to the physical properties of the granules. The following observations and experiments clarify the finding to some extent.

In the three experiments summarized in Table I, a marked decrease in the dry weight of the mitochondrial fractions occurred when the granules were washed in saline. Additional observations have shown that the loss in dry weight is considerably greater and more rapid if the washing is conducted in hypotonic media, such as water or dilute buffers. Furthermore, when simply suspended in water, the granules can be observed to swell enormously and eventually to disintegrate. If the water suspension is allowed to stand for several days at 4°, only a small fraction of the original

TABLE II

Comparison of Aerobic Succinoxidase and Anaerobic Succinic Dehydrogenase Activities of Large Granules and Microsomes

Preparation	Experiment No.	Succinic dehydrogenase $\frac{Q_{N_2}}{Q_{CO_2}}$	Succinoxidase Q_{O_2}	Ratio, $Q_{N_2} : Q_{O_2}$
Large granules (washed 3 times)	1	205	63	3.3
	2	251	70	3.6
	3	229	62	3.7
Microsomes (washed 2 times)	1	29	7	4.1
	2	30	4	7.5
	3	36		

large granules can be recovered by low speed centrifugation, the remaining material consisting of soluble substances, including protein, and a particulate component which is of considerably smaller size than the original mitochondria (12, 13).

The results given in Table III illustrate the rate of decline in the succinoxidase activity of large granules kept in isotonic and hypotonic media, and demonstrate that the stability of the enzyme system is considerably greater in the former than in the latter type of medium. One is led to assume that either the dehydrogenase or an unknown component of the system, intermediate between the dehydrogenase and cytochrome *c*, was lost through lability or dilution.

In another experiment, a preparation of large granules was washed twice with saline, suspended in hypotonic $NaHCO_3$ (0.025 M), and tested both aerobically and anaerobically for activity against succinate. When retested after standing for 72 hours at 4°, the preparation had lost over 90 per cent of its aerobic succinoxidase activity but only 28 per cent of its original anaerobic succinic dehydrogenase activity. The particulate

material of the aged preparation was then isolated by centrifugation at $18,000 \times g$ for 1 hour, washed twice in 0.025 M NaHCO_3 , and finally resus-

TABLE III
Effect of Hypotonic Media on Succinoxidase Activity of Large Granules

Experiment No.	Tonacity	Composition of medium	Per cent of original succinoxidase activity after standing	
			24 hrs.	48 hrs.
1	Isotonic	0.85% NaCl	100	66
	Hypotonic	H ₂ O	50	44
2	Isotonic	0.85% NaCl containing 0.01 M phosphate, pH 7.4	77	68
	Hypotonic	0.01 M phosphate pH 7.4	46	44
3	Isotonic	0.85% NaCl containing 0.025 M NaHCO ₃	96	37
	Hypotonic	0.025 M NaHCO ₃	34	7

TABLE IV

Effect of Hypotonic Medium on Aerobic Succinoxidase and Anaerobic Succinic Dehydrogenase Activity of Large Granules

Preparation	Aerobic determination		Anaerobic determination	
	Per ml. preparation per hr.	Q_{O_2}	Per ml. preparation per hr.	Q_{N_2/CO_2}
L.G. ₀ (original preparation in 0.025 M NaHCO_3)	c.mm. O ₂ 420	70	c.mm. CO ₂ 2035	340
L.G. ₁ (L.G. ₀ after standing 72 hrs. at 4°)	17	3	1470	245
L.G. ₂ (L.G. ₁ recovered by centrifugation and resuspended)	<10	<2*	875	142
S (supernatant after centrifugation of L.G. ₁)			38	10
L.G. ₃ (L.G. ₂ washed twice and resuspended)			58	30
L.G. ₃ + S			518†	267†

* The cytochrome oxidase Q_{O_2} for preparation L.G.₂ was 360 c.mm. of O₂ per mg. per hour. The low succinoxidase activity of the preparation was therefore not a result of loss of cytochrome oxidase content.

† These values are calculated on the basis of the aliquot of L.G.₃ tested. The aliquots of both L.G.₃ and S were the same as those employed when the two preparations were tested separately.

pended in the same medium. The activity of the various preparations is shown in Table IV.

The following points of interest can be derived from the data presented in Table IV. (1) The results of the anaerobic measurements indicate that

the succinic dehydrogenase system is considerably more stable in hypotonic media than would appear from assays of the complete succinoxidase system. (2) The anaerobic succinic dehydrogenase activity of the sedimentable material remaining after treatment of large granules with a hypotonic bicarbonate solution can, however, be reduced to low levels by repeated washing in the same medium (L.G.₁ → L.G.₂ → L.G.₃). (3) A soluble fraction (S), obtained after lysis of large granules in hypotonic bicarbonate

TABLE V

Effect of p-Dimethylaminoazobenzene on Distribution of Cytochrome Oxidase and Succinoxidase in Cytoplasmic Extract of Rat Liver

Experiment No.	Diet	Liver fraction	Cytochrome oxidase		Succinoxidase	
			Q ₀	Per cent recovery	Q ₀	Per cent recovery
1	<i>p</i> -Dimethyl-aminoazobenzene, 36 days	Extract	32	(100)	7.5	(100)
		Large granules (washed 3 times)			46	35
		Microsomes (washed 2 times)			<2	<2
2	<i>p</i> -Dimethyl-aminoazobenzene, 50 days	Extract	33	(100)	7.8	(100)
		Large granules (washed 3 times)	228	48	29	25
		Microsomes (washed 2 times)	<5	<1	2	2
3	Control diet, 44 days	Extract	24	(100)	6.7	(100)
		Large granules (washed 3 times)	243	54	60	47
		Microsomes (washed 2 times)	<10	<2	4	3
4	Control diet, 56 days	Extract	37	(100)	9	(100)
		Large granules (washed 3 times)	234	63	55	50
		Microsomes (washed 2 times)	<5	<1	3	2

solution, contains a substance which greatly enhances the succinic dehydrogenase activity of the lysed and washed particulate material (L.G.₃).

Effect of Ingestion of p-Dimethylaminoazobenzene—Table V presents the results of four experiments designed to determine the effect of *p*-dimethylaminoazobenzene on the cytochrome oxidase and succinoxidase activity of the cytoplasmic extract and its two particulate components. The conditions in the four experiments listed in Table V are comparable except that the animals in Experiments 1 and 2 received *p*-dimethylaminoazobenzene added to the basic diet.

The livers of the animals that received the carcinogenic compound showed no gross lesions. The only change noted microscopically in representative stained sections was a wide variation in the size of the parenchymal cells and their nuclei, but in no instance was cirrhosis or tumor formation detected. The livers of the animals that received the control diet appeared grossly and microscopically normal.

The data of Table V, in the light of those obtained with the liver of rats fed a normal diet (Table I), show that neither the butter yellow nor the control diet appreciably affected the cytochrome oxidase and succinoxidase activity of the liver extract. The activity (Q_{O_2}) and per cent recovery of both enzyme systems in the mitochondrial fractions derived from the animals fed the control diet (Experiments 3 and 4, Table V) were, however, slightly less than comparable values recorded in Table I. In Experiment 2, Table V, in which the large granules obtained from animals fed butter yellow were assayed for cytochrome oxidase, neither the Q_{O_2} nor the per cent recovery of this enzyme was greatly different from comparable values obtained in Experiments 3 and 4 with the control animals. The succinoxidase Q_{O_2} and per cent recovery values obtained in one butter yellow experiment (Experiment 1, Table V) were somewhat lower than the control values and in the next butter yellow experiment (Experiment 2, Table V) were considerably lower than the control values.

DISCUSSION

Intracellular Distribution of Enzymes—The observations reported in the present paper lead to the conclusion that most, if not all, of the cytochrome oxidase and succinoxidase activity of the cytoplasmic extract is associated with relatively large granules, approximately 0.5 to 2 μ in diameter. The findings supporting this conclusion can be summarized as follows: (1) When both the large granule and microsome fractions were removed from the extract, the supernatant S_2 showed no demonstrable cytochrome oxidase or succinoxidase activity. (2) An average of 70 per cent of the former and 74 per cent of the latter enzyme system was recovered in the mitochondrial fraction. (3) Only a small proportion of the activity of the extract (less than 4 per cent of the cytochrome oxidase and approximately 7 per cent of the succinoxidase) was found in the microsomes. Although the activity of the microsomes was appreciable, it was probably accounted for by the presence of large granules or of large granule fragments in the microsome fractions. In this respect, direct observations of the latter preparations in the dark-field microscope indicated that the number of large granules present was probably sufficiently high to account for the enzymatic activity of the microsomes. (4) When the over-all recovery of enzyme originally present in the extract is considered, it should be noted

that two inhomogeneous fractions, Sd_1 and Sd_2 , were discarded. Sd_1 consisted essentially of agglutinated large granules and a small number of nuclei and red blood cells, and Sd_2 of a mixture of large granules and microsomes. On the basis of dry weight measurements and microscopical observations, it has been estimated that the number of large granules present in the discarded fractions is usually 10 to 20 per cent of those originally present in the extract. Furthermore, occasional determinations have shown that the discarded large granules possess roughly the same enzyme activity as that of the main large granule fraction. The amount of activity, in terms of recovery, which can be accounted for as present in large granules thus approaches 90 per cent for both enzyme systems.

Taken together, these observations suggest that the cytochrome oxidase and succinoxidase systems, when isolated by centrifugation from the cytoplasm of the liver cells of the rat, are entirely localized in the so called large granules. This point is of considerable importance since it indicates that, in the living cell itself, these cytochrome-linked enzyme systems are situated in corresponding cytoplasmic elements.

The fact that the two enzyme systems under study are associated with insoluble components of the cell has been well known for many years and has been frequently alluded to in the literature both as a point of fundamental biological significance and as the primary reason that neither system is well understood. As early as 1913, Warburg (6) noted that the large granules were responsible for most of the oxygen uptake exhibited by cell-free extracts of guinea pig liver. Bensley and Hoerr (18) were able to separate the large granule fraction by means of centrifugation, and concentrates thus obtained were shown by Lazarow and Barron (8, 9) to possess succinoxidase activity. The latter investigators, however, were unable to find any significant difference in the activity of two particulate fractions separated by differential centrifugation. Stern (7), working with heart muscle, demonstrated that particles 50 to 200 $m\mu$ in diameter were associated with both cytochrome oxidase and succinoxidase activity. In the present experiments the distribution of the cytochrome oxidase and succinoxidase systems has been placed on a quantitative basis, and both systems have been shown to be associated, probably exclusively, with granules of a definite range of size.

Problem of Cytological Identification of Particulate Fractions—Although the exact nature of the large granules is still unsettled, microscopical observations indicate that in the present experiments the large granule fraction consisted for the most part of those cytoplasmic inclusions known under the term mitochondria. Evidence for this view, which has been reviewed by Claude (12, 13), can be summarized as follows: Of the particulate components present in the cytoplasm of the normal liver cell that can

be seen in either fixed or unfixed preparations, only mitochondria and secretory granules possess a range in size comparable to that of isolated large granules. Particulate glycogen and microsomes, on the other hand, are of sufficiently smaller size to permit effective separation from the large granules by differential centrifugation. The abundance of secretory granules in the intact liver cell and, presumably, in the cytoplasmic extract can be varied considerably by simple alteration of the food intake of the animal; viz., when the animals are fasted, secretory granules accumulate in great numbers in the liver cells, whereas in animals fed normally to the time of death the liver cells contain very few secretory granules. In the present experiments with normal rats (Table I) the animals were given an ample supply of food, and the proportion of secretory granules present in the large granule fraction isolated by centrifugation was probably small.

A possible objection to the belief that the large granule fraction consists for the most part of mitochondria is based on the observation that isolated large granules are uniformly spherical, whereas the mitochondria of liver cells are frequently elongated. It is well known, however, that injuries of various sorts to the cell will cause mitochondria to become spherical. This can be demonstrated, for example, by observing cultured cells under dark-field illumination as they rupture and their mitochondrial content disperses in an isotonic medium.

It is, of course, tempting to believe that the mitochondria of the living cell contain all of the cytochrome oxidase and succinoxidase present in the cytoplasm and therefore, that the mechanism by which molecular oxygen is utilized in the cytoplasm of the cell can be ascribed in large measure, if not entirely, to a definite morphological entity. Before this view can be definitely accepted, however, the relation between the large granules of the extract and mitochondria must be further clarified.

Work from this laboratory has indicated that the microsome substance represents the chromophilic component of the ground substance of cytoplasm (19). Centrifugation experiments (19) and electron microscope studies of cultured cells (20) and of sections of guinea pig liver (21) support the view that the microsome material exists in the cell in the form of particulate, submicroscopic units. The part played in the economy of the cell by the microsomes is at present unknown.

Components of Succinoxidase System—A number of observations, several of them already mentioned, have shown that the large granules can be affected by changes in the tonicity of the medium, probably through the existence of a limiting membrane, and that their disintegration in hypotonic media is accompanied by a rapid and pronounced loss in aerobic succinoxidase activity. By the use of the ferricyanide method, it has been possible to follow more directly the changes affecting this cytochrome-linked enzyme

during disintegration of the granules. Thus, it was found that the anaerobic succinic dehydrogenase activity was largely retained in a hypotonic medium even though the aerobic activity had almost disappeared (Table IV) and that the anaerobic activity could be reduced to low levels only by repeated washing of the granules in hypotonic medium. This apparent difference in stability is difficult to explain on the basis of the present data. It is suggested, however, that the sharp decline in aerobic activity with time may be largely a dilution phenomenon, whereby substances intermediate in the chain of hydrogen transfer between succinate and cytochrome *c* are released from the granules to such an extent that their concentration relative to the cytochrome oxidase system is reduced below a critical level. Such a dilution effect might be expected to occur more readily in a reaction dependent on cytochrome oxidase than in a reaction involving ferricyanide, since cytochrome oxidase is firmly bound to particulate material, whereas ferricyanide is present in solution in great excess.

The data in Table IV also demonstrate the existence of a soluble component of the succinic dehydrogenase system, a finding which confirms experimental results reported previously (7, 22). More recently (23), it has been found that the intact succinic dehydrogenase system can be obtained in a soluble form through treatment of the large granules with acetone and subsequent extraction of the residue with 0.01 M NaHCO₃. Further studies dealing with the properties of the dehydrogenase will be reported at a later date.

Effect of p-Dimethylaminoazobenzene on Succinoxidase Activity of Liver Fractions—The preliminary data presented in Table V indicate that the ingestion of *p*-dimethylaminoazobenzene over a period of less than 2 months produces a considerable reduction in the succinoxidase content of the washed large granules but has little, if any, effect on the succinoxidase content of the liver extract. Although an explanation for this finding must await further experimentation, it seems possible that the large granules obtained from rats fed the carcinogenic substance were abnormally fragile and therefore allowed the escape during the washing procedure of an unusually large amount of a soluble component of the succinoxidase system.

SUMMARY

1. A study of the distribution of two enzyme systems, cytochrome oxidase and succinoxidase, in the cytoplasm of the liver cell of the rat has been presented. A liver extract, containing only cytoplasmic material and suitable for fractionation by differential centrifugation, was utilized as an experimental tool.

2. Both enzyme systems were shown to be associated, probably exclusively, with sedimentable granules of a relatively large size (0.5 to 2 μ in diameter). A smaller sedimentable component (60 to 150 m μ in diameter),

which was present in large amount in the liver extract, showed little, if any, enzyme activity and the remaining soluble material of the extract showed no activity. A number of observations suggested that the enzymatically active large granule fraction consisted chiefly of those cytoplasmic inclusions known as mitochondria.

3. The large granules were markedly affected by changes in tonicity of the surrounding medium, their disintegration in hypotonic media being accompanied by a rapid and pronounced loss in aerobic succinoxidase activity. This decline in succinoxidase could not be explained either by loss of cytochrome oxidase activity, which remained at a high level, or, from the results of anaerobic determinations, by loss of succinic dehydrogenase itself. By repeated washing of the large granules in a hypotonic medium, it was possible, however, to reduce their anaerobic succinic dehydrogenase activity to a low level, apparently through the release of a soluble component of the enzyme system.

4. The ingestion over a period of less than 2 months of the carcinogenic substance, *p*-dimethylaminoazobenzene, produced a reduction in the succinoxidase content of the large granules of rat liver.

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THE STABILITY OF STREPTOMYCIN*

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The biological inactivation of streptomycin in acid and alkaline solution was first noted by Waksman and his coworkers (1). In a recent review, Waksman and Schatz (2) reported that solutions of crude streptomycin maintained their original potency during 15 to 17 days at 37°, but at 100° about 50 per cent of the activity was lost in 10 minutes. However, the pH of the solutions was not indicated. Other investigators concerned with degradation studies have described the products obtained by acid and alkaline hydrolysis of streptomycin. Folkers *et al.* (3-5) and Carter *et al.* (6) have shown that, on acid hydrolysis, streptomycin is cleaved into two basic fractions, streptidine, 1,3-diguanido-2,4,5,6-tetrahydroxycyclohexane (7, 8), and streptobiosamine. On acid hydrolysis the latter compound yields N-methyl-L-glucosamine (5), and an alkaline hydrolysis of streptidine yields streptamine, 1,3-diamino-2,4,5,6-tetrahydroxycyclohexane (7, 8).

The rapid inactivation of streptomycin in *N* sodium hydroxide was indicated by Carter *et al.* (9). When the hydrolysis of streptomycin hydrochloride was carried out in *N* sodium hydroxide at 100° or for a longer period at 40°, a substance was isolated and characterized as maltol (10).

The present investigations were carried out to determine the effect of pH and temperature on commercial and pure streptomycin sulfate. The ranges covered in these experiments are summarized in Table I. Although streptomycin in aqueous solution is sensitive to acids, bases, and heat, it has been found that its optimum stability lies between pH 3 and 7, at temperatures at or below 28°.

EXPERIMENTAL

Materials and Method

Preparation of Pure Streptomycin Sulfate—Commercial streptomycin hydrochloride, 500 γ per mg., was dissolved in methanol and treated with an excess of a methanol solution of calcium chloride. After evaporation *in vacuo*, the streptomycin hydrochloride-calcium chloride double salt was allowed to crystallize at room temperature. The crude double salt was

* Presented before the Division of Medicinal Chemistry at the meeting of the American Chemical Society at Atlantic City, April, 1946.

filtered and recrystallized from methanol. After drying it for 48 hours at 25° and 30 μ , 9.9 per cent methoxyl and 1.8 per cent water were found by the Karl Fischer reagent method. However, the double salt was converted to streptomycin sulfate and the aqueous solution was concentrated *in vacuo* to a low volume; it was lyophilized and further dried at 26° and 50 μ over barium oxide for 2 days; activity 860 γ per mg. (*Bacillus subtilis* plate assay¹); $[\alpha]_D^{25} = -79^\circ$ (c, 1 in water).

Analysis— $(C_{21}H_{39}N_7O_{12})_2 \cdot (H_2SO_4)_1$

Calculated. C 34.61, H 5.81, N 13.46, SO₄ 19.78

Found. " 34.34, " 6.02, " 13.28, " 19.50

TABLE I

Half Life of Pure Streptomycin under Conditions of pH and Temperature (t in Hours)

pH	7°	28°	50°	95°
0.8	1200	110	8	
1.7	Stable	1500	90	
2.7	"	Stable	990	
5.5	"	"	4600	37
7.0	"	"		
8.6	"	1100	50	
9.5	3000	300	28	
11.2		16		

Streptomycin sulfate (1.144 gm.) was dissolved in 75 ml. of water and cooled to 5°. The solution was treated with the calculated amount of 0.420 N solution of barium hydroxide and immediately titrated with 0.1067 N sulfuric acid at 5°, with a glass electrode. All the readings were corrected for temperature. Approximate pK values were obtained from the mid-points of titration (Fig. 1) which occur at pH 4.5 and 9.5. 3 equivalents of sulfuric acid were required for complete neutralization of the streptomycin.

Two series of observations were made with use of (a) this pure streptomycin sulfate and (b) commercial clinical streptomycin sulfate (Pfizer) (400 γ per mg.). Weighed quantities of both purified and partly purified materials were dissolved in solutions of the desired pH to give concentrations of approximately 1000 γ per ml. Streptomycin solutions of both series were stored in 100 ml. ground glass-stoppered volumetric flasks. A small quantity of toluene was added to each flask to prevent the growth

¹ This assay was obtained by comparison with a standard related to the original Waksman standard.

of microorganisms. The solutions were then raised to the desired temperature and a zero time sample (in duplicate) was withdrawn with a calibrated pipette. The samples for bioassay were transferred directly into phosphate buffers at pH 6.5 to retard inactivation of streptomycin.

When no buffer is used in solutions of sodium hydroxide at pH 8 to 10, the alkali is consumed and the streptomycin loses potency at a decreasing rate with a corresponding drop in pH. In this work, streptomycin sulfate

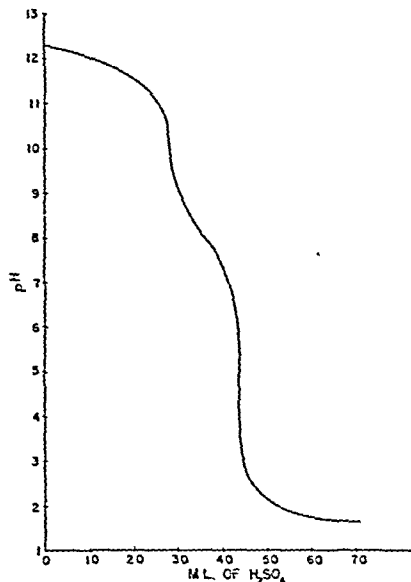


FIG. 1. Acid titration of streptomycin

was dissolved in phosphate buffers in regions at and above pH 8. All the solutions were checked periodically with a Cambridge pH meter.

The lowest temperature at which measurements were made was afforded by a refrigerator at $7^{\circ} \pm 0.3^{\circ}$. A constant temperature bath was used for the 23° and 50° observations and a well insulated electric oven was employed for the temperature at $95^{\circ} \pm 0.2^{\circ}$.

Assays—The plate assays were carried out essentially by the method of Schmidt and Moyer (11) which was used for penicillin, but with the following exceptions: (a) The medium used was Difco Bacto-streptomycin assay agar, originally employed by Skell (unpublished) for the assay

of streptothricin; (b) *Bacillus subtilis*, American Type Culture Collection No. 9524, was used as the test organism; (c) a 1 per cent phosphate buffer at pH 7.9 to 8.0 was used.

Turbidimetric values were obtained by a modification of the method of McMahan (12) for penicillin assays. The test culture employed was a strain of *Escherichia coli* supplied by Dr. S. A. Waksman.

The *Bacillus subtilis* cylinder plate method gave more precise assays with less deviation than the *Escherichia coli* turbidimetric method throughout the whole course of these studies; consequently only *Bacillus subtilis* assay values were used to calculate the velocity constants of inactivation.

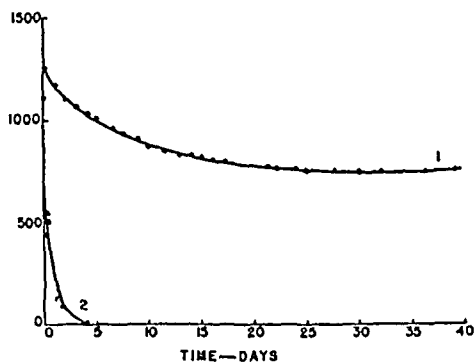


FIG. 2. Inactivation of commercial streptomycin with time at pH 6.6. Curve 1 at 50°; Curve 2, at 95°. *Bacillus subtilis* plate assay. Ordinate, streptomycin in micrograms per ml.

Results

Conditions of Stability—There appears to be no inactivation of either pure streptomycin sulfate or of commercial streptomycin (400 γ per mg.) at concentrations of approximately 1000 γ per ml. during at least 60 days in the range of pH 3 to 7 at 7° and 28°.

Inactivation at Elevated Temperatures—Whereas streptomycin solutions are relatively stable in the range of pH 3 to 7 at or below room temperature, the rate of inactivation of commercial streptomycin becomes appreciable at more elevated temperatures, as is shown in Fig. 2. At 50° and pH 6.6 about 33 per cent of a sample of partially purified streptomycin was destroyed at the end of 15 days. More drastic decomposition took place at 95°, at which 50 per cent of the streptomycin was destroyed in 4.5 hours. Solutions of the pure normal salt of streptomycin sulfate (pH 5.5) at similar concentrations (1000 γ per ml.) are more stable at these temperatures, as is shown in Fig. 3.

Inactivation by Acid—The acid hydrolysis of streptomycin into the two basic fractions streptidine and streptobiosamine below pH 2 was found to

be a pseudounimolecular reaction by application of the first order equation,

$$k = \frac{1}{t} \ln \frac{c_0}{c_0 - c} \quad (1)$$

where c_0 is the initial concentration of streptomycin and c is the decrease

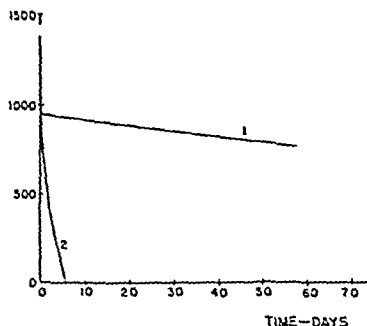


FIG. 3. Inactivation of pure streptomycin sulfate (pH 5.5) with time at 50°, Curve 1, and at 95°, Curve 2. *Bacillus subtilis* plate assay. Ordinate, streptomycin in micrograms per ml.

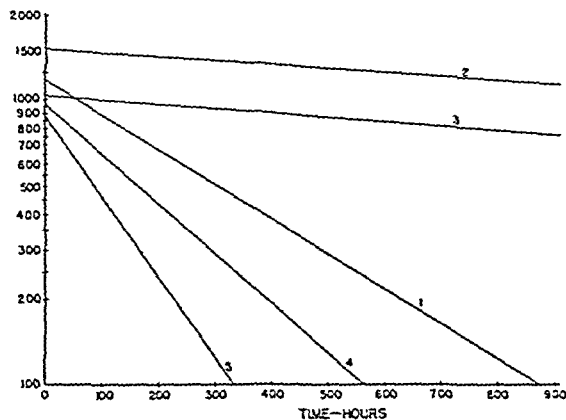


FIG. 4. Hydrolysis of streptomycin with time at 23°. Curve 1, partially purified streptomycin at pH 1; Curve 2, partially purified streptomycin at pH 2; Curve 3, pure streptomycin at pH 1.7; Curve 4, pure streptomycin at pH 0.95; Curve 5, pure streptomycin at pH 0.80. Ordinate, streptomycin in micrograms per ml.

after a lapse of time, t . When the concentration of streptomycin is plotted against time, exponential curves are obtained which when plotted on semi-logarithmic paper produce straight lines (within experimental errors), as is shown in Figs. 4 and 5. The velocity constants (k hour⁻¹) of inactiva-

tion of partially purified streptomycin sulfate (400 γ per mg.) at concentrations of about 1000 γ per ml. and at pH 1.0 were found to be 0.0025 at 28°, and 0.040 at 50°; at pH 2.0, 0.00043 at 28°, and 0.0043 at 50°. For the purified material at pH 0.8 k hour⁻¹ was found to be 0.00056 at 7°, 0.0065 at 28°, and 0.090 at 50°; at pH 1.7, 0.00045 at 28° and 0.0080 at 50°; at pH 2.7, 0.0007 at 50°. The rate of hydrolysis in acids is essentially the same for pure and partially purified streptomycin.

Inactivation by Alkali—The inactivation studies in the alkaline region were carried out in buffers in the range of pH 8.0 to 11.4 between 7° and 50°.

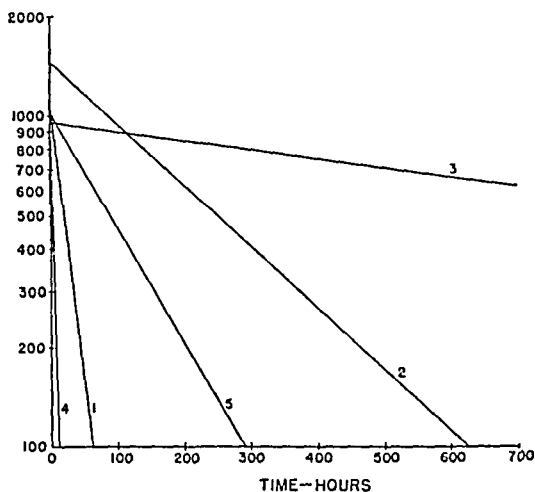


FIG. 5. Hydrolysis of streptomycin with time at 50°. Curve 1, partially purified streptomycin at pH 1; Curve 2, partially purified streptomycin at pH 2; Curve 3, pure streptomycin at pH 2.7; Curve 4, pure streptomycin at pH 0.8; Curve 5, pure streptomycin at pH 1.7. Ordinate, streptomycin in micrograms per ml.

The pH of these solutions remained constant during the course of the observations. The velocity constants obtained by alkali inactivation on different samples of commercial streptomycin sulfate were not reproducible; however, when the concentration of pure streptomycin is plotted against time, the curves in the alkaline range are similar to those obtained in acid medium. The data fall on a straight line when they are plotted on semi-logarithmic paper (Fig. 6). The reaction rate constants calculated from the first order equation (No. 1) in the alkaline range for purified material k hour⁻¹, was found to be, at pH 8.6, 0.00065 at 28°; at pH 9.5, 0.00023 at 7°, 0.0023 at 28°, and 0.024 at 50°; at pH 11.2, 0.044 at 28°.

A more complete summary of the data obtained in this work is given in Table I where the half life of purified streptomycin under conditions of pH and temperature is indicated.

Stability of Dry Streptomycin Salts—Salts of commercial streptomycin containing less than 1 per cent moisture have been shown to be stable at or below room temperature over long periods of time. No drop in potency has been noted in material which has been at laboratory temperature for 1 year. Some recent observations were made on both streptomycin hydro-

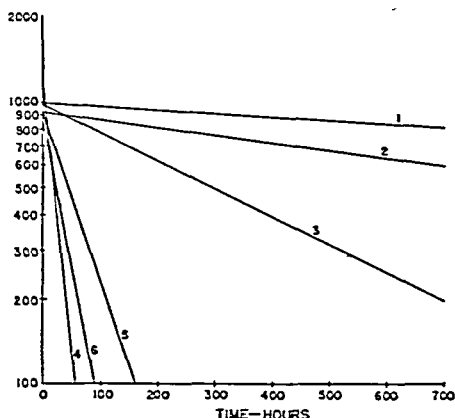


FIG. 6. Inactivation of pure streptomycin with time. Curve 1, 7°, pH 9.5; Curve 2, 28°, pH 8.6; Curve 3, 28°, pH 9.5; Curve 4, 28°, pH 11.2; Curve 5, 50°, pH 8.6; Curve 6, 50°, pH 9.5. Ordinate, streptomycin in micrograms per ml.

TABLE II
Stability of Dry Streptomycin Salts; Bacillus subtilis Plate Assay

Streptomycin	Per cent moisture	Original potency	After 2 wks. at 50°	After 5 wks. at 50°	After 10 wks. at 50°
		γ per mg.	γ per mg.	γ per mg.	γ per mg.
Hydrochloride.....	0.86	249	238	245	243
Sulfate.....	0.68	393	403	405	387
"	0.89	391	376	384	366

chloride and sulfate at 50° for a period of 10 weeks. These stability data are given in Table II.

SUMMARY

The most favorable conditions for the stability of solutions of streptomycin are at temperatures at or below 28° and between pH 3 and 7. Inactivation takes place outside of this range; namely, below pH 3 and above pH 8. The velocity constants of inactivation have been determined in

both acid and alkaline regions and the reactions have been found to be irreversible and first order. In the acid region the velocity constants of the partially purified material are essentially the same as for pure streptomycin. Heat greatly increases the rate of decomposition over the whole pH scale.

Relatively dry neutral salts of streptomycin are stable at 50° over long periods.

The authors wish to thank Mr. J. R. McMahan for the microbiological assays and Mr. F. H. Hedger for the data on the dry salts of streptomycin.

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A FURTHER INVESTIGATION OF THE RÔLE OF BETAINE IN TRANSMETHYLATION REACTIONS IN VIVO*

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The fact that betaine is so far the only N-methyl compound, other than choline, which is capable of supporting growth on a homocystine diet (1), has led us to investigate further its rôle in transmethylation. The metabolic fate of the methyl groups and the glycine portion of the molecule have been simultaneously followed by labeling the methyl groups with deuterium and the glycine moiety with N¹⁵.

Two growing rats were fed isotopic betaine chloride (55 mg. per rat daily) on an otherwise methyl-free diet for 2 weeks. Choline and creatine were isolated from the tissues and their deuterium and N¹⁵ concentrations were determined. Glycine and glutamic acid were also isolated from the tissue proteins for the determination of their N¹⁵ concentrations.

At the end of the experimental period, more than 40 per cent of the methyl groups of choline and more than 25 per cent of the methyl groups of creatine had been derived from the betaine fed. On the other hand, only 0.5 per cent of the nitrogen of these compounds had been derived from the dietary betaine. In fact, the N¹⁵ concentration of isolated glycine from the tissue proteins was about twice as high as that of the isolated choline and creatine.

When deuteriodimethylglycine was fed to growing rats in amounts equivalent in methyl groups to 55 mg. of betaine chloride for a period of 3 weeks, the amount of deuterium which appeared in the methyl groups of choline and creatine was negligible. It was less than 10 per cent of the amount which resulted after a 2 week feeding of betaine. Confirmatory evidence of the inefficiency of dimethylglycine as a methyl donor was obtained by testing its ability to prevent the formation of hemorrhagic kidneys. It proved to be completely inactive in this respect.

EXPERIMENTAL

Synthesis of N¹⁵ Glycine—Isotopic glycine was synthesized from chloroacetic acid and ammonium carbonate in a manner similar to that described

* The authors wish to thank the Nutrition Foundation, Inc., for the research grant which has aided this work.

for the preparation of glycine by Cheronis and Spitzmueller (2). Since the N^{15} was available in the form of ammonium nitrate ($N^{15}H_4NO_3$), it was necessary to convert the compound to ammonium carbonate. A concentrated solution of 1.8 moles of the isotopic ammonium nitrate (approximately 5 atom per cent excess N^{15} in the ammonium N) was added dropwise into a distilling flask containing solid sodium hydroxide (1.8 moles). As is indicated in Fig. 1, a stream of nitrogen was passed through flask A which was warmed gently, and then the stream of gas was passed through a con-

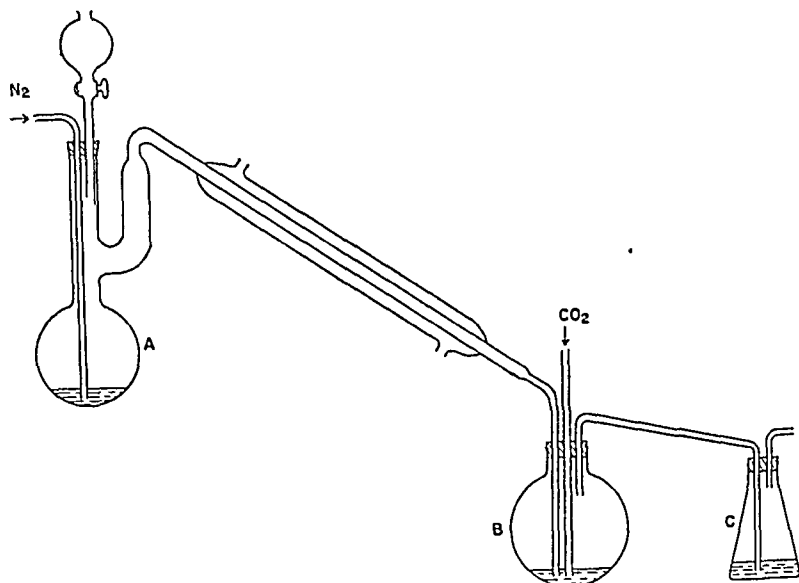


Fig. 1. Apparatus used in the preparation of N^{15} glycine

denser into flask B containing 75 cc. of water cooled to 0° . The nitrogen stream was further led through trap C which contained sulfuric acid and was designed to absorb any ammonia which was not absorbed by the water in flask B. A stream of carbon dioxide was simultaneously passed through flask B until no further absorption took place. After all of the ammonium nitrate had been added, the solution in flask A was boiled until half the volume had been distilled. Flask B, containing the solution of ammonium carbonate, was then disconnected and 20 gm. of chloroacetic acid (approximately 95 per cent pure) were added. The solution was allowed to stand for 24 hours at room temperature and then the excess ammonia was distilled into the sulfuric acid in trap C. The residual solution was treated with norit, 200 cc. of methanol were added, and the solution was allowed to stand in the refrigerator for 2 days. The crude product was filtered and suspended for 2 hours in 50 cc. of 90 per cent methanol. Upon filtration,

10.7 gm. of product were obtained. The glycine was resuspended in 50 cc. of 90 per cent methanol for 2 hours and filtered. The amount of glycine obtained was 9.9 gm.

Calculated, N 18.7; found, N 18.5

The recovery of the isotopic nitrogen was excellent; of the N^{15} starting material, 75 per cent was recovered in the sulfuric acid solution as ammonium sulfate which could be utilized directly in the repetition of this synthesis or in other preparations, 15 per cent in the methanol filtrates, and 8 per cent in the glycine itself.

Synthesis of N^{15} -Deuteriobetaine Chloride $((CD_3)_2N^{15}ClCH_2COOH)$ —1.7 gm. of isotopic glycine were dissolved in 80 cc. of 2 N sodium hydroxide in a round bottom flask. After the alkaline solution had been aerated for $1\frac{1}{2}$ hours to remove the small amount of ammonia which was present as a contaminant, 10 gm. of deuteriomethyl iodide (3) were added and the flask was stoppered securely. The reaction vessel was shaken in a water bath at 65–70° until the methyl iodide layer had disappeared, and the solution was then allowed to remain at this temperature for 1 hour.

The alkaline reaction mixture was cooled and, after acidification to Congo red with HCl, was treated with an aqueous solution of ammonium reineckate and allowed to remain in the refrigerator overnight in order to precipitate all the betaine as betaine reineckate. The betaine reineckate was filtered off, washed with dilute HCl, and dried in air for several hours. The salt was then dissolved in dilute NH_4OH solution and was decomposed with Ag_2O , freshly prepared from 5.1 gm. of $AgNO_3$. The silver reineckate was removed by filtration and was washed thoroughly with water. The combined filtrate and washings were heated to approximately 60° and were aerated to remove the ammonia. The excess Ag_2O which precipitated was removed by filtration, and the solution so obtained was concentrated *in vacuo* to a small volume which was then acidified with HCl. The $AgCl$ which precipitated was removed by filtration. The filtrate was then concentrated to dryness *in vacuo*. The residue was extracted repeatedly with a total of 200 cc. of boiling absolute ethanol. Betaine chloride precipitated from the extract which had been allowed to stand in the refrigerator. The salt was filtered, and washed first with cold absolute ethanol and then with ether. 1.47 gm. of product were obtained. By concentration of the mother liquors, a second crop of 0.64 gm. was obtained.

Elementary. Calculated,¹ N 8.70; found, N 8.67

Isotopic. Deuterium 61.9 ± 0.6 atom % excess

N^{15} 5.20 ± 0.003 atom % excess

¹ All calculated values are based on increased molecular weight due to deuterium in the molecule.

Synthesis of Deuteriodimethylglycine $((\text{CH}_2\text{D})_2\text{NCH}_2\text{COOH})$ —Glycine was methylated with formaldehyde and deuterioformic acid by a method described by Clarke, Gillespie, and Weisshaus (4). Deuterioformic acid (DCOOD) was prepared by thermal decomposition of deuteriooxalic acid, $(\text{COOD})_2$, which had been prepared by exchange of anhydrous oxalic acid with D_2O by the method used in the preparation of deuteriodimethylaminoethanol (5). From 55 gm. of oxalic acid, a yield of 20 gm. of formic acid, DCOOD , was obtained in 40 cc. of aqueous solution. 12 gm. of trioxymethylene were dissolved completely in the solution and then 12 gm. of glycine were added. The temperature was cautiously raised to the boiling point and the mixture was refluxed for 10 hours. After the reaction mixture had been cooled and acidified with HCl , the volatile compounds were removed by distillation. Water was added repeatedly and subsequently removed by distillation in order to replace all exchangeable deuterium from the dimethylglycine hydrochloride. The product was recrystallized twice from glacial acetic acid and was washed with acetone. 6.7 gm. of dimethylglycine hydrochloride were obtained, m.p. 187° (uncorrected).

Elementary. Calculated, $\text{N } 10.0$; found, $\text{N } 9.99$

Deuterium. 13.49 ± 0.10 atom % excess

Feeding Experiments

Feeding of Isotopic Betaine—A pair of young rats, one male and one female, weighing 71 and 59 gm. respectively, was used as experimental animals. During the feeding period, the rats received *ad libitum* a synthetic choline-free diet in which homocystine was the only sulfur-containing amino acid. The basal methyl-free diet had the following percentage composition: amino acid mixture 21.3 (6), salt mixture (Osborne and Mendel (7)) 4.0, agar 2.0, dextrin 25.7, sucrose 15.0, corn oil (Mazola) 30.0. To 98 parts of the basal diet were added 1.25 parts of *dl*-homocystine and 0.75 part of dextrin. The oil-soluble vitamins were included in the corn oil as previously described (6).

The water-soluble vitamins were administered in aqueous solution. Each rat received daily two 0.5 cc. portions of a solution containing 0.01 mg. each of thiamine chloride, pyridoxine hydrochloride, riboflavin, and nicotinic acid, 0.1 mg. of *dl*-calcium pantothenate, and 12.5 mg. of choline-free ryzamin-B (1).

Isotopic betaine chloride was fed at a level equivalent in methyl groups to 50 mg. of choline chloride per rat daily. Each rat, therefore, received 55 mg. of isotopic betaine chloride neutralized with 20 mg. of NaHCO_3 per day in 1 cc. of the solution containing the water-soluble vitamins.

At the end of 2 weeks, the animals were sacrificed, and choline and creatine were isolated from the tissues (3). The isolated choline chloroplatinates and creatinine potassium picrates were analyzed for deuterium and N^{15} . Prior to the analyses for N^{15} , the picric acid was removed from the creatinine potassium picrate by continuous extraction with ether.

Choline chloroplatinate

Elementary. Rat 1746. Calculated,¹ Pt 31.3; found, Pt 31.4

" 1747. " " 31.3; " " 31.3

Isotopic. " 1746. 22.0 ± 0.4 atom % excess D; 0.105 ± 0.003
atom % excess N^{15}

Rat 1747. 21.5 ± 0.2 atom % excess D; 0.089 ± 0.003
atom % excess N^{15}

Creatinine potassium picrate

Deuterium. Rat 1746. 5.51 ± 0.17 atom % excess

" 1747. 5.81 ± 0.08 " % "

Creatinine

N^{15} . Rat 1746. 0.072 ± 0.003 atom % excess

" 1747. 0.072 ± 0.003 " % "

Glycine and glutamic acid were also isolated from the combined tissue proteins. These amino acids were isolated from the tissues after they had been freed of fat in the course of the procedure for the isolation of choline and creatine. The fat-free tissues were extracted three times with cold 0.6 per cent trichloroacetic acid. The proteins were then dried by washing first with boiling alcohol, then with ether, and finally were hydrolyzed in 20 per cent hydrochloric acid. From the hydrolysate, glutamic acid was isolated as the hydrochloride (8).

Glutamic acid hydrochloride

Rat 1752. Calculated, N 7.63; found, N 7.41

" 1753. " " 7.63; " " 7.64

" 1755. " " 7.63; " " 7.46

" 1757. " " 7.63; " " 7.67

Glycine was precipitated from the protein hydrolysate as the trioxalatochromiate and converted to carbobenzoxyglycine by the procedure described by Bergmann and Niemann (9).

Carbobenzoxyglycine

Rat 1752. Calculated, N 6.70; found, N 6.41

" 1753. " " 6.70; " " 6.53

" 1754. " " 6.70; " " 6.62

" 1755. " " 6.70; " " 6.53

The N^{15} concentrations of the isolated amino acids are given in Table I.

Feeding of Deuteriodimethylglycine—One male and one female rat, weighing 65 and 59 gm. respectively, were fed deuteriodimethylglycine hydro-

chloride at a level equivalent in methyl groups to 50 mg. of choline chloride per rat daily. Each rat received daily 75 mg. of deuteriodimethylglycine hydrochloride neutralized with NaHCO_3 . It was found necessary to give this solution by stomach tube. The 1.25 per cent homocystine diet and the vitamin supplements were identical with those used in the preceding feeding experiment with isotopic betaine.

TABLE I

Feeding Experiments with Isotopic Betaine for 2 Week Period
0.77 gm. of betaine chloride ingested.

Rat No. and sex	Change in body weight	Compound isolated	Deuterium in methyl group (A)	Per cent methyl groups derived from betaine ($A/C^* \times 100$)	N^{15} (B)	Per cent N derived from betaine ($B/D^\dagger \times 100$)
	gm.		atom per cent excess		atom per cent excess	
1746 ♀	59-75	Choline chloroplatinate	34.2 ± 0.6	41.5 ± 0.7	0.105 ± 0.003	2.02 ± 0.06
		Creatinine K picrate	22.0 ± 0.7	26.7 ± 0.8	0.17^\ddagger	3.2^\ddagger
		Glycine			0.203 ± 0.003	3.90 ± 0.06
		Glutamic acid			0.024 ± 0.003	0.46 ± 0.06
1747 ♂	71-89	Choline chloroplatinate	33.4 ± 0.3	40.5 ± 0.4	0.089 ± 0.003	1.71 ± 0.06
		Creatinine K picrate	23.2 ± 0.3	28.2 ± 0.4	0.17^\ddagger	3.2^\ddagger
		Glycine			0.190 ± 0.003	3.66 ± 0.06
		Glutamic acid			0.025 ± 0.003	0.48 ± 0.06

* $C = 82.5$ (atom per cent excess D in betaine methyl groups).

† $D = 5.20$ (atom per cent excess N^{15} in betaine).

‡ Value for N^{15} in glycine moiety calculated from the observed value of total N of creatine (0.72 ± 0.003) on the basis of isotopic N distribution data of Bloch and Schoenheimer (10) obtained by feeding N^{15} glycine to growing rats.

On the 11th day of the experimental period, the kidneys of both rats appeared to be enlarged and it was observed that the animals had lost weight rapidly during the preceding 4 day period. Therefore, a single dose of 25 mg. of choline chloride was administered to each animal on the 11th day. On the 12th day, feeding of the experimental diet was resumed and was continued for the duration of the experiment. The animals were sacrificed on the 21st day and autopsy revealed yellow blotches on the kidneys and indications of fatty degeneration of the liver in both rats. Choline and creatine were isolated in the usual manner from the tissues and were analyzed for deuterium.

Choline chloroplatinate

Elementary.	Rat 1197.	Calculated, ¹ Pt 31.7; found, Pt 32.2
	" 1198.	" " 31.7; " " 31.7
Deuterium.	" 1197.	0.63 \pm 0.06 atom % excess D
	" 1198.	0.38 \pm 0.06 " % " "

Creatinine potassium picrate

Deuterium.	Rat 1197.	0.11 \pm 0.05 atom % excess D
	" 1198.	0.12 \pm 0.08 " % " "

The data from this experiment are summarized in Table II.

Effect of Dimethylglycine on Development of Hemorrhagic Kidneys—The procedures and diet used in this test have been described in a preceding

TABLE II

Feeding Experiment with Deuteriodimethylglycine for 3 Week Period

1.15 gm. of dimethylglycine ingested.

Rat No. and sex	Change in body weight	Choline isolated		Creatine isolated	
		Deuterium in methyl groups (A)	Per cent methyl groups derived from dimethylglycine (A/B* \times 100)	Deuterium in methyl group (C)	Per cent methyl group derived from dimethylglycine (C/B* \times 100)
	gm.	atom per cent excess		atom per cent excess	
1197 ♂	65-60	0.98 \pm 0.09	4.4 \pm 0.4	0.44 \pm 0.20	2.0 \pm 0.9
1198 ♀	59-58	0.59 \pm 0.09	2.6 \pm 0.4	0.48 \pm 0.32	2.1 \pm 1.4

* B = 22.5 (atom per cent excess D in methyl groups of dimethylglycine).

paper (5). Two groups of eight rats were used; one group was kept on the basal diet (5) as controls, while the other group was given the basal diet supplemented by dimethylglycine at a level of 1 per cent of the diet. The changes in body weight, the average daily food consumption, and the incidence of hemorrhagic kidneys in each group are given in Table III.

DISCUSSION

Betaine occurs in only very small amounts in animal tissues (11). However, its activity as a labile methyl compound suggests that it may play an important rôle in the animal organism. Betaine, like choline, supports growth of young rats on homocystine diets (12), it is a lipotropic agent (13), and it prevents the development of hemorrhagic kidneys (14). However, both growth tests (12) and hemorrhagic kidney experiments (14) have indicated a quantitative difference between betaine and choline. In the growth experiments (12) with young rats, this difference was seen principally in a delay of several days in the initiation of growth following the administration of betaine to methyl-deficient animals. Once growth had started, there appeared to be no significant difference between the effective-

ness of betaine and that of choline as measured by growth rate.² On the other hand, the hemorrhagic kidney experiments of Griffith and Mulford (14) indicated that, at low concentrations in the diet, betaine is only about one-third as effective as choline. From these results, they concluded that "either the three betaine methyls are poorly utilized or that only one of the three is a labile methyl."

TABLE III

Effect of Dimethylglycine on Development of Hemorrhagic Kidneys

Average daily food intake per rat, 4.3 gm.

	Average body weight			Average kidney weight	Incidence of renal lesions, No. of rats
	Initial	6th day	8th day		
	gm.	gm.	gm.	gm.	
Dimethylglycine (8 rats)	37.3	49.6	48.4*	1.01*	5 Severe 2 Partial 1 Normal
Controls (8 rats)	35.4	47.5	46.3†	0.98†	5 Severe 2 Partial 1 Normal

* Average for seven rats; one rat found dead on the morning of the 8th day.

† Average for seven rats; one rat died on the 7th day.

The experiments with N¹⁵ deuteriobetaine have revealed betaine to be an extremely efficient methyl donor for the synthesis of choline and creatine. The rate at which methyl groups from dietary betaine appear in tissue choline is of the same order as the rate at which the methyl groups from dietary choline appear in tissue choline (15). It is evident from a comparison of the deuteriomethyl and N¹⁵ contents of the tissue choline (Table I) that the nitrogen of betaine is not utilized directly in the conversion to choline. The nitrogen of betaine appears rapidly in the glycine of the tissue proteins. This observation is in agreement with the data of Stetten (8) who showed that the nitrogen of betaine finds its way to choline by way of glycine and aminoethanol. Stetten's experimental results taken in conjunction with our earlier findings that the methyl groups of betaine could be utilized in transmethylation led to the scheme presented by Stetten (8) wherein the methyl groups and the nitrogen of the betaine molecule arrive in the choline by different reaction paths. The present experiments with both the nitrogen and the methyl groups of betaine labeled offer direct proof of this scheme.

² It should be noted that in the experiment with N¹⁵ deuteriobetaine the test animals had been growing on a diet containing adequate choline prior to the administration of isotopic betaine, and that growth continued uninterrupted after the feeding of the isotopic compound was started.

The methyl groups of dietary methionine appear more rapidly in creatine (3) than do those of dietary betaine. Borsook and Dubnoff have found that methionine (16) can serve as a methyl donor in the enzymatic synthesis *in vitro* of creatine from guanidoacetic acid, but that choline can function in this system only in the presence of homocystine (17). Thus, it may well be that the transfer of methyl groups from choline and also betaine to creatine involves transmethylation first to methionine and then either directly or through one or more intermediary steps to creatine.

In contrast to betaine, dimethylglycine proved to be a very poor source of labile methyl groups for choline and creatine. The relative inefficiency of dimethylglycine as a methyl donor was foreshadowed by its inability to support growth of young rats on an otherwise methyl-free, homocystine-containing diet (18). As is shown in Table II, over a period of 3 weeks the deuteriodimethylglycine provided only a very small percentage, about 3.5, of the methyl groups of choline and an even smaller percentage, about 2, of those of creatine. Nevertheless, in view of the observation that dimethylglycine not only fails to support growth of rats on homocystine diets (18), but also does not prevent the development of hemorrhagic kidneys, it is of interest that some transmethylation apparently did take place. It is likely that the amount of transmethylation that does occur is much too small to prevent the development of renal lesions. From these data, it also appears that dimethylglycine is not appreciably converted to betaine.

SUMMARY

Betaine labeled with deuteriomethyl groups and N^{15} was synthesized and fed to growing rats. Isotopic analyses of the choline and creatine isolated from the rat tissues showed betaine to be an extremely effective methyl donor. Methyl groups from dietary betaine appear in tissue choline almost as rapidly as they appear from dietary deuteriocholine. The disparity in amounts of N^{15} and of deuterium found in the tissue demonstrates conclusively that the betaine molecule is not converted as a whole to choline.

Dimethylglycine containing deuterium in the methyl groups was fed to young rats. Transmethylation from this compound to choline and to creatine occurred to only a very small degree. Dimethylglycine also was tested for its activity in preventing the development of renal lesions and was found to be without effect.

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THE PYRIDOXINE CONTENT OF TISSUES OF RATS FED VARIOUS DIETS

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In 1941 McHenry and Gavin (1) reported that pyridoxine was necessary for the synthesis of fat from protein in rats and suggested that pyridoxine was essential for the metabolism of protein. The observations of Bernhard, Steinhauser, and Matthey (2) were in agreement. Cerecedo and Foy (3) found that pyridoxine deficiency in rats could be accelerated in onset and increased in severity by maintenance of the animals on a high protein diet. More conclusive proof of the relation of pyridoxine to protein metabolism has been obtained recently by the observations of Schlenk and Snell (4), and of Gunsalus *et al.* (5), relating the vitamin to enzyme systems.

It seemed advantageous to study pyridoxine retention in rats fed various diets in order further to ascertain the relation of the vitamin to intermediary metabolism. Mitchell and Isbell (6) determined the pyridoxine content of normal rat tissues, but not in relation to diet. Wright *et al.* (7) found that the concentration of pyridoxine in normal rats was greatest in the liver and kidney, and also concluded that the amount in any tissue was relatively constant if the animal had been fed a reasonably adequate diet. Results reported below offer a modified interpretation.

Method

All animals used were albino rats of the Wistar strain, raised in the colony of the Connaught Medical Research Laboratories. They were housed in individual, screen bottom cages and were provided with water and food *ad libitum*. Details of diet are given for each experiment. All diets contained vitamins A and D in adequate amounts; B vitamin supplements were administered by subcutaneous injection in amounts noted elsewhere. In all cases young rats having initial weights of 70 to 100 gm. were used. One-half of each experimental group was male, the other half female, and no attempt was made to measure the effect of sex upon vitamin content.

At the conclusion of the feeding experiments the rats were killed by stunning and the pyridoxine content of tissues was determined by the microbiological procedure of Atkin, Schultz, Williams, and Frey (8). This method gives the total amount of pyridoxine, pyridoxal, and pyridoxamine

present, calculated as pyridoxine hydrochloride, and was found to be, as judged by recovery experiments, sufficiently accurate.

EXPERIMENTAL

To study the effects of varying proportions of dietary carbohydrate, fat, and protein, three types of diets were used. The compositions of the three diets are given in Table I.

TABLE I
Composition of Diets, Per Cent

	Carbohydrate diet	Fat diet	Protein diet
Cane sugar.....	84	54	0
Casein.....	10	10	96
Lard.....	0	20	0
Corn oil.....	0	10	0
Agar.....	2	2	0
Salt mixture*.....	4	4	4
Cod liver oil concentrate.....	0.015	0.015	0.015

*(Steenbock-Nelson, Salts 40).

TABLE II
Average Pyridoxine Content of Rat Tissues

Dietary treatment	Pyridoxine content, γ per gm. wet tissue		
	Liver	Kidney	Muscle
Carbohydrate diet plus vitamin B ₆	7.5	9.4	6.8
“ “ minus “ B ₆	5.1	4.9	4.1
Fat diet plus vitamin B ₆	7.0	7.1	6.4
“ “ minus “ B ₆	5.6	3.3	4.2
Protein diet plus vitamin B ₆	15.5	7.0	6.2
“ “ minus “ B ₆	5.8	4.3	4.0

Six groups of rats, nine to twelve animals in each group, were used. Two groups were placed on each diet and rats in one of each such group were given 40 γ of pyridoxine hydrochloride per day by injection. Rats in the other group on each diet received no pyridoxine. All rats were given 25 γ of thiamine chloride, 100 γ of calcium pantothenate, 25 γ of riboflavin, 100 γ of niacin, and 10 mg. of choline chloride per rat per day. Animals were thus maintained for 21 days.

Data regarding the average pyridoxine content of liver, kidney, and leg muscle are given in Table II. As would be expected, the amount in tissues of rats not given pyridoxine is less than in animals receiving the vitamin.

The greatest difference in concentration was observed in rats supplied with pyridoxine and a high protein diet. In this case the concentration in the liver was increased markedly.

In rats maintained on an intermediate, and more normal, level of protein, the average concentration of pyridoxine in the liver was found to be 10.6 γ per gm. The diet used was fox chow (Toronto Elevators) and it contained 20 per cent protein. The average supply of protein and of pyridoxine secured per rat per day was approximately the same as in Experiment 1. As judged from three values (those obtained in Experiments 1 and 2), there seems to be a linear relation between the proportion of protein in the diets and the pyridoxine storage in the livers of rats given the vitamin.

To determine whether the pyridoxine content of liver and kidney could be altered by supplying increasing amounts of the vitamin to groups of rats maintained on a constant protein intake, four groups of animals with the same average initial weight were kept on a diet of fox chow for 3 weeks. The total average amounts of pyridoxine obtained by rats in each of the four groups were 60, 68, 88, and 2060 γ per rat per day. Average food intakes for each group were about the same and the weight gains of the four groups showed no significant difference. The pyridoxine content of the livers and of the kidneys was not significantly different for the four groups and was similar to the value reported in Experiment 2. The administration of a large excess of pyridoxine caused no increased storage in the two tissues.

Because of the results in the first three experiments, it was decided to conduct a series in which the effects of two variables (protein proportion in the diet and pyridoxine intake) could be compared. For this purpose forty male and forty female rats were randomly divided into twenty groups of four animals each. Four diets were employed. These had constant amounts of four constituents (corn oil 10 per cent, agar 2 per cent, salt mixture 4 per cent,¹ and cod liver oil concentrate 0.015 per cent). Varying quantities of casein and sucrose were included. The percentages of casein were 10, 20, 40, and 80, and of sucrose 74, 64, 44, and 4. Five groups of rats were maintained on each diet and the pyridoxine intake varied for the five groups as follows: 0, 25, 50, 100, and 200 γ per rat per day. Consequently, there were five levels of pyridoxine intake for each diet and there were four types of diet for each level of pyridoxine supply. Animals were maintained on the regimen for 3 weeks. They were then killed and the pyridoxine content of each liver was determined. The results are given in Table III.

Weight increases, shown in Table IV, were examined statistically. There was no significant difference between the average weight changes of groups

¹Steenbock, H., and Nelson, E. M., *J. Biol. Chem.*, 56, 362 (1923).

receiving 20 and 40 per cent protein at any one level of pyridoxine intake, and at these protein levels the average weight changes were about the same for all groups which received pyridoxine, although weight increases of rats without pyridoxine were significantly less. Rats on an 80 per cent protein diet showed about the same weight changes as did those on a 10 per cent level, regardless of the pyridoxine supply, and such changes were less than those shown by animals on intermediate proportions of protein.

TABLE III

Pyridoxine Content of Rat Livers in Experiment 4, Micrograms per Gm. of Wet Tissue

Pyridoxine hydrochloride intake, γ per rat per day	Protein in diet			
	10 per cent	20 per cent	40 per cent	80 per cent
0	5.33	4.70	5.00	4.10
	3.97	4.06	6.53	4.11
	3.53	5.01	6.89	3.93
	4.56	2.98	5.06	3.93
25	6.54	12.06	11.65	13.26
	7.78	5.97	10.04	11.64
	8.86	20.94	11.62	15.54
	15.83	6.96	9.42	14.32
50	10.80	12.29	7.90	10.50
	8.75	11.51	8.83	14.16
	8.59	10.24	17.18	22.19
	9.44	11.34	13.07	21.47
100	10.87	7.62	4.83	12.20
	6.85	10.77	13.94	15.88
	11.66	14.41	18.86	21.48
	8.69	12.92	16.70	18.48
200	13.25	14.47	4.93	20.30
	10.94	19.43	13.25	27.78
	6.82	12.42	11.15	19.20
	7.74	3.70	12.14	16.72

An analysis of variance of the pyridoxine content of the liver due to various intakes of pyridoxine was made. The pyridoxine content of the livers of animals not receiving pyridoxine was significantly less than all other values. For all other values there was shown to be a significant variation due to protein intake, but not to the dosage of pyridoxine. The variation due to protein proportion in the diet was linear, confirming the indication obtained in Experiments 1 and 2.

To determine the effect of subsistence on a diet devoid of protein, twelve male and twelve female rats were randomly divided into two groups, one of which received 40 γ of pyridoxine per rat per day, while pyridoxine was

not supplied to the other group. All animals were given the following diet: sucrose 77, lard 10, corn oil 7, salt mixture 4,¹ agar 2, and cod liver oil concentrate 0.015 per cent.

All animals received the B vitamin supplements recorded in the description of Experiment 1. The regimen was continued for 2 weeks, when the animals were killed.

There was no significant difference in weight gains between the two groups. The group which received pyridoxine had an average liver content of 7.1 γ per gm. of wet tissue, while in the other group the average liver pyridoxine was 5.5 γ . The difference between the two groups was found to be significant at the 5 per cent level. The liver content was in good agreement with values predicted from the relations between liver content, pyridoxine, and protein intakes, found in Experiment 4.

TABLE IV
Average Weight (Gm.) Increases of Rats in Experiment 4

Pyridoxine hydrochloride intake, γ per rat per day	Protein in diet			
	10 per cent	20 per cent	40 per cent	80 per cent
0	20	142	143	65
25	132	279	275	135
50	120	288	287	135
100	131	297	271	87
200	152	283	283	99

DISCUSSION

The experimental data indicate that in rats to which an external source of pyridoxine is not available for 3 weeks the residual concentration in the liver, kidney, and leg muscle is independent of the protein intake. If there is a dietary supply of pyridoxine, the concentration in the liver increases in direct proportion to the percentage of protein in the diet. The pyridoxine content of the liver can be increased by increasing the pyridoxine intake up to 25 γ per rat per day, if the percentage of protein in the diet remains constant; it can also be increased by increasing the proportion of protein in the diet, if the intake of pyridoxine is constant. On all levels of protein proportion which were tried up to 80 per cent of the diet, 25 γ of pyridoxine per rat per day appeared to be sufficient to give a liver concentration which was maximal for a particular protein supply.

When the supply of protein is in the range generally used in feeding experiments (10 to 20 per cent of the diet), the liver concentration of pyridoxine is not greatly different for different diets and, in this limited

respect, the present results are in agreement with the general conclusion of Wright *et al.* (7) that the diet had little effect.

When rats are deprived of pyridoxine for 3 weeks, concentrations in the liver, kidney, and leg muscle are approximately equal. Prior to the experimental work it was assumed that subsistence on a high protein diet would cause a greater depletion of the pyridoxine content of the tissues because of a need for pyridoxine in the catabolism of amino acids. Such was not the case, but the feeding of a high protein diet caused a large increase in liver pyridoxine when the vitamin was supplied. In these observations the only marked difference between rats supplied and not supplied with pyridoxine was seen in the animals on a high protein diet.

Several inferences could be drawn from the results of the above experiments. Apparently, an increase in dietary protein causes a greater storage of pyridoxine in the liver. It could be suggested that the augmented storage is caused by an increased need for pyridoxine in the liver to care for a greater volume of amino acid catabolism. This suggestion is in accordance with the various observations relating pyridoxine to protein catabolism. Another inference is that, in studies of pyridoxine function, it would be advantageous to compare tissues of rats with and without pyridoxine, fed on a diet rich in protein. Such studies might advance more rapidly knowledge of the mechanism of pyridoxine action.

The independence of tissue concentration to intakes of pyridoxine hydrochloride greater than 25 γ per day indicates that the requirement of rats for this vitamin is less than this amount. Dimick and Schreffler (9) found that rat requirement on a diet containing 27 per cent protein was 10 γ per day.

SUMMARY

The concentration of pyridoxine in rat liver appears to be independent of pyridoxine supply when the intake is greater than 25 γ per day, but is directly proportional to the percentage of protein in the diet. The liver content of pyridoxine can be significantly increased by supplying the vitamin to rats maintained on a high protein diet. The result offers a helpful approach to a study of pyridoxine function.

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PHOSPHORUS COMPOUNDS IN THE GASTROCNEMIUS MUSCLES OF SCORBUTIC GUINEA PIGS

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Scurvy is characterized by languor and incapacity for work. Whether these symptoms are consequences of inanition or due to some metabolic defect is not known. The possible rôle of ascorbic acid in the general problem of fatigue has only recently received notice. Brack (4) has reported that additions of ascorbic acid delay the onset of fatigue of isolated muscle for several hours. According to Basu and Ray (2), on the basis of their studies with the finger ergometer, the contractibility of a muscle and its fatigability depend on the condition of its saturation with vitamin C. Additional studies on isolated muscle suggested that vitamin C augments contraction, brings about quicker relaxation, and delays the onset of fatigue (1). Crandon *et al.* (5) in their comprehensive study of a subject on a vitamin C-deficient diet for 6 months showed that, while there was a definite inability to perform aerobic work, the capacity for anaerobic work was undiminished.

Giri's report (7) that ascorbic acid is one of the substances in tissue which plays an important rôle in the regulation of the activity of tissue phosphates suggests that the phosphorus compounds involved in muscular contraction may be involved in the ready fatigability of scorbutic individuals. Furthermore, Berg (3) has shown that vitamin C prevents or hinders the reduction of adenylyltri-phosphoric acid observed in heart muscle following thyroxine administration. Several investigators (11, 12) have also reported a decreased phosphocreatine content in the muscle of guinea pigs in the terminal stages of vitamin C deficiency. However, no complete study of all the compounds involved in muscular contraction during the development of scurvy has been made. In the course of other studies of vitamin C it was consistently noted that there was a fairly wide zone of latent deficiency in which there was a marked loss of body tone.

It was felt that the investigation of phosphorus compounds during the entire stage of onset of scurvy might reveal physiological impairments resulting from a moderate deficiency not directly associated with the usual symptoms of a complete or more drastic vitamin deficiency.

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Methods

Guinea pigs 6 to 7 weeks of age and weighing from 250 to 300 gm. were separated into small groups and housed in clean wire cages. The animals were fed a scorbutigenic diet suggested to us by Dr. O. A. Bessey.¹ This diet was adequate in all respects and was capable of allowing not only growth but also normal reproductive activity. After 2 weeks on this diet, when all animals were gaining weight, the ascorbic acid was deleted from the diet of some of the animals. Animals fed the scorbutigenic diet but no ascorbic acid invariably evidenced clinical signs of scurvy within 20 to 24 days by extensive hemorrhages and fragility of the bones (15). Histological confirmation of scurvy was obtained on the costal junctions of the ribs.

Normal and scorbutic animals were sacrificed at intervals during a period of 32 days. The guinea pigs, after fasting for 12 hours, were anesthetized with nembutal. A gastrocnemius muscle was dissected free, leaving nerves and major blood vessels intact, and frozen *in situ* with carbon dioxide snow. The procedure of sampling this muscle and the description of methods employed for the determination of solids, nitrogen, creatine, glycogen, lactic acid, and various phosphate compounds have been given previously (9). Blood for determination of lactate, sugar, and ascorbic acid was obtained by cardiac puncture. Plasma ascorbic acid determinations were made by the macromethod of Mindlin and Butler (10).

Results

The data are presented in Tables I, II, and III. The results of analyses on the nine control animals (Table I) are presented individually to illustrate the extent of variability encountered in those substances for which analyses were performed. In general, the variations observed are of a relatively minor nature.

Thirty-four guinea pigs were used in following the course of developing scurvy (Table II). Although the majority of the animals were sacrificed during the latter half of the period on the scorbutic diet, sufficient animals were used during the early period to give an indication of the degree of alteration occurring during the onset of scurvy.

No changes in the concentrations of either muscle or blood lactates were noted at any time. During the first half of the dietary period the guinea pigs maintained their weight or even gained in weight. However, in the following days a rapid decrease in weight was noted, the average decrease

¹ Scorbutigenic diet: soy bean, autoclaved, 36 per cent; rolled oats, 25 per cent; skim milk powder (cooked 3 hours), 20 per cent; alfalfa meal (cooked 3 hours), 8 per cent; peanut oil, 5 per cent; CaCO_3 , 1 per cent; NaCl , 1 per cent; brewers' yeast, 4 per cent; 2 ml. of cod liver oil to each animal per week, 30 mg. of ascorbic acid dissolved in water and fed by dropper to each animal per week.

being approximately 17 per cent. Greater changes were observed in the latter stages.

The total phosphorus in the gastrocnemius muscle decreased slightly but was not consistent (Table II). On the other hand the acid-soluble phosphates and various subportions of this fraction exhibited a striking change. In those animals maintained on the scorbutic diet up to 15 to 16 days, the average concentration of acid-soluble phosphorus was similar to control values. From this time on the concentration diminished; the

TABLE I

Analyses of Gastrocnemius Muscle of Guinea Pig

The phosphorus is expressed in mg. per 100 gm. of tissue.

Guinea pig No.	Total phosphorus	Acid-soluble P	Inorganic P	Phospho-creatine	Adenosine tri-phosphate	Hexose phosphate	Barium-insoluble phosphate	Solids	Nitrogen	Creatine	Glycogen	Lactate	
												Muscle	Blood
								per cent	gm. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
1	220	159	22	57	51	22	79	21.8	3.0	403	0.51	27	
2	229	164	18	55	66	22	84	22.4	3.8		0.31	53	58
3	229	177	35	58	52	20		21.9	3.0	398	0.51	29	10
4	254	151	23	63	31	26	70	22.1	3.2	378	0.91	18	24
5	245	160	13	58	56	23	65	22.6	2.4	470	0.50	15	15
6	202	160	19	59	48	19	77	22.7	3.2	403	0.34	23	19
7	213	143	15	58	39	23	68	22.5	2.9	488	0.29	24	
8	220	142	13	57	47	16	55	22.2	2.9	425	0.63	15	14
9	227	157	27	52	47	15	63	22.3	2.9	429	0.74	25	18
Average.	227	157	21	59	49	21	70	22.3	3.0	425	0.53	26	23

average decrease for the animals during the last half of the scorbutic dietary period was approximately 21 per cent. This decrease was reflected in all of the components of this fraction in a somewhat proportionate manner. Phosphocreatine, adenosine triphosphate, and the barium-insoluble phosphates also exhibited diminishing concentrations during the last 16 days on the diet, roughly averaging 25 per cent. The fall in hexose phosphate phosphorus was less marked and more irregular.

The slight decrease in the solid matter of the gastrocnemius muscle was accompanied by a diminishing protein content. Muscle creatine decreased roughly to the same extent as the phosphocreatine phosphorus, indicating the close interrelationship of these two compounds. Glycogen was extremely variable but in the latter phases of the deficiency diet values above 400 mg. per cent were seldom observed.

It must be emphasized that a constantly diminishing concentration of all the compounds determined was not observed. Individual variations were

TABLE II

*Analyses of Gastrocnemius Muscle of Guinea Pig on Progressive Days
of Scorbatic Diet*

The phosphorus is expressed in mg. per 100 gm. of tissue.

Day of deficient diet	Percent- age change in weight	Total P	Acid-soluble P	Inorganic P	Phospho- creatine	Adenosine tri- phosphate	Hexose phos- phate	Barium- insoluble phosphate	Solids	Nitrogen	Creatine	Glycogen	Lactate	
													Muscle	Blood
									per cent	gm. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
1st	+2	223	158	27	58	52	18	80	21.7	2.7	359	0.99	86	80
4th	+8	261	180	25	61	50	36	89	21.7	2.4	404	0.99	23	
7th	+4	238	138	21	50	48	22	67	22.2	2.3	437	0.86	18	11
8th	+4	248	160	34	36	33	18	101	22.2	2.3	444	0.81	32	68
9th	+14	188	169	21	61	33	21	73	23.0	3.2	399	0.40	15	10
10th	+8	245	162	23	52	34	17	84	22.7	2.9	418	0.44	25	7
12th	+1	207	160	10	60	41	19		21.7	3.2	453	0.56	19	17
13th	+2	264	168	23	59	38	37	85	22.1	2.3	478	0.27	24	8
14th	-7	250	160	14	39	65	14	80	22.7	3.0	402	0.69	23	52
15th	+8	234	179	15	62	36	20	90	22.5	3.1	423	0.66	19	16
16th	+1	239	163	26	53	40	19	87	22.2	3.1	403	0.93	25	14
17th	-10	205	160	28	47	38	20	84	21.8	2.8	395	0.63	26	9
18th	-15	217	140	12	38	34	18	66	21.7	2.9	341	0.65	29	17
20th	0	218	150	26	45	39	14	79	20.9	2.1		0.52	24	7
20th	-10	207	123	14	34	42	14	69	21.1	2.8	278	0.25	28	15
21st	-20	220	112	16	39	41	10	50	23.0	2.3	147	0.44	31	10
21st	-21	212	119	10	53	30	22	44	20.7	1.9	268	0.18	28	26
21st	+7	184	106	25	30	26	20	60	19.8	1.9	384		10	11
22nd	-9	244	137	6	53	40	32	47	20.5	1.9	338	0.14	25	21
22nd	-23	244	137	27	28	44	24	90	23.5	2.3	359		63	71
22nd	-21	242	146	12	50	34	49	48	19.2	2.5	416	0.54	17	9
22nd	-20	200	105	9	48	33	39	31	21.2	2.7	334	0.17	22	9
23rd	-20	240	114	18	33	52	37		22.2	2.2	425		29	
23rd	-22	185	171	19	43	32	22		17.6	2.5	363		24	23
24th	-24	203	149	25	35	24	15	76	20.1	2.0	373	0.18	59	76
25th	-16	248	129	21	35	30	18	59	21.0	1.6	350	0.70	26	31
25th	-14	199	112	29	33	44	13	75				0.19		30
28th	-18	224	137	30	45	27	34	85	22.2	1.5	388	0.41	15	10
29th	-19	180	110	8	34	19	42	35	19.0	2.1	331	0.22	36	10
29th	-19	215	97	8	35	14	19	27	21.5	2.3	225	0.16	37	13
30th	-21	201	130	13	55	36	17	44	19.7	2.1	353	0.40	24	14
31st	-22	216	130	15	41	37	47		22.3	3.2	339	0.22	5	10
31st	-28	232	121	10	39		49	76	22.6	2.2	248	0.37	28	26
32nd	-32	181	110	8	33	36	9	51	21.7	3.2	274	0.22	30	21

present and, while it was not possible to correlate this exactly with more obvious signs of the degree of deficiency, it appeared that some animals

were more resistant and did not develop scurvy to the same extent as other guinea pigs, which were on the diet for identical periods of time.

The data presented in Table III were obtained from five guinea pigs which, on the 22nd day of the diet, exhibited clinical signs of scurvy. They were then given 100 mg. of ascorbic acid daily and sacrificed. The concentrations of glycogen acid-soluble phosphates and the subfractions only were determined. It appeared that ingestion of at least 500 mg. of ascorbic acid was necessary before changes in these compounds began to appear. The concentrations of creatine and phosphocreatine showed the most prompt return to normal values. Additional work is planned in an attempt to clarify this point.

TABLE III

Effects of Scorbatic Diet Followed by Vitamin C on Guinea Pig Gastrocnemius Muscle

All the values are expressed in mg. per 100 gm. of tissue. 100 mg. of ascorbic acid were given daily after the 22nd day on the deficient diet.

Guinea pig No.	Ascorbic acid	Creatine	Total acid-soluble P	Inorganic P	Phospho-creatine	Adenosine triphosphate	Hexose phosphate	Barium-insoluble phosphate
	mg.							
1	100	271	145	25	23	33	13	70
2	200	372	125	22	35	38	22	70
3	300	280	124	22	31	40	18	76
4	500	443	167	17	54	33	18	82
5	600	347	152	30	49	30	30	72

DISCUSSION

During the first 16 days on the scorbutigenic diet, the gastrocnemius muscle showed only minor variations in the concentrations of the various compounds under study (Table II). After this period of time we found diminished concentrations which continued to decrease at a rapid rate for the remainder of the test period. It was interesting that the guinea pigs began to lose weight during this same interval. Semistarvation, therefore, might be considered the factor mainly responsible for the decreased concentrations which occurred. However, tests conducted on animals which were placed on a submaximal dietary intake showed that this was not the case. These results will be reported in detail later. Nevertheless, it is significant that the first positive clinical evidence of scurvy began to appear after the first 16 days on a scorbutigenic diet and correlates very well with the changes in those compounds that were found to be diminished during the final stages of scurvy.

Although the increase in the water content of muscle was not very large, it was quite consistent and tends to confirm the findings of Doi (6) and of

Sheppard and McHenry (13). This latter group of investigators had also reported decreases in the protein content of the entire carcass of approximately the same magnitude observed to occur in the gastrocnemius muscle of the guinea pigs in this study. The concentrations of glycogen, phosphocreatine, creatine, adenosine triphosphate, and a number of other acid-soluble phosphorus compounds were reduced. Since all of these compounds are involved in one way or another in the complex reactions occurring during muscular contraction, their simultaneous reduction in the scorbutigenic state is highly significant.

Several authors (8, 11, 12, 14), working on guinea pigs in the terminal stages of vitamin C deficiency, have reported decreases of large magnitude in the phosphocreatine content of the muscles. However, these reduced values were accompanied by large increases in either lactic acid or in inorganic phosphorus. These changes are generally indicative of a failure to maintain resting conditions in the animal at the time of sampling and, consequently, doubt as to the validity of the results is inevitable. In the experiments that were reported in this paper there was no evidence of increased concentrations of either lactic acid or inorganic phosphorus. The values were well within the limits generally obtained for quiet, resting animals. Therefore, the changes that were observed to begin after about 16 days on the scorbutigenic diet are suggestive and could explain the weakness that develops in guinea pigs prior to the appearance of symptoms of frank scurvy. The low levels of phosphocreatine observed and interference with its synthesis can readily account for the general weakness and fatigability observed in scorbutic guinea pigs.

When vitamin C was given to scorbutic guinea pigs (Table III), phosphocreatine rapidly returned to near normal concentrations. No increase in the animals' weights had occurred at this time. Muscle glycogen did not increase and, therefore, while these results confirmed the increase in phosphocreatine following vitamin C ingestion first reported by Rat-simamanya and his coworkers (8, 12), our results failed to substantiate their report of increases in glycogen and decreases in muscle lactate.

In sharp contrast to the lowering of the acid-soluble phosphate of muscle obtained from scorbutic guinea pigs was the maintenance of total phosphorus at practically constant levels. This would tend to confirm the frequent reports that despite the decalcification of bone in scorbutic animals there is no apparent derangement of calcium-phosphorus metabolism. More important, however, it makes clear that, unless complete studies of all phases of metabolism are studied, erroneous impressions can be obtained. Derangement of phosphorus metabolism does occur, since the acid phosphates are diminished in amount. Consequently, there was an absolute increase in the concentration of acid-insoluble or lipoidal phosphates of muscle. Sheppard and McHenry (13) observed that scorbutic guinea

pigs have a tendency, despite loss of body weight, to retain body fat. How these facts are related to the reduction of alkaline phosphates in scurvy requires additional investigation. Studies on metabolism of the lipid phosphates will help to elucidate the rôle played by vitamin C in tissue metabolism.

SUMMARY

Studies have been made on the concentrations of a number of compounds in the gastrocnemius muscle of the resting guinea pig during the development of scurvy. Major changes occurred after the animals had been on the scorbutigenic diet for approximately 16 days, this time coinciding roughly with the first appearance of clinical signs of scurvy.

Phosphorus metabolism was primarily affected. Total phosphorus of the muscle was diminished slightly but the acid-soluble phosphates were lowered dramatically. The reductions in the various acid-soluble phosphorus compounds were in a fairly direct ratio to the decrease in the total acid-soluble phosphorus.

Glycogen, total nitrogen, creatine, and muscle solids were also reduced, but in varying degrees.

The anorexia that accompanied depletion of the guinea pig's store of vitamin C appeared to be related to the diminished concentrations in muscle of certain compounds concerned with muscular contraction. The low levels of phosphocreatine and the interference with its synthesis (note also the low levels of creatine and adenosine triphosphate) readily explain the general weakness and ready fatigability observed in these animals. Addition of ascorbic acid to the diet of scorbutic guinea pigs rapidly restored the concentrations of phosphocreatine to nearly normal levels.

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ISOLATION AND PROPERTIES OF IMMUNE LACTOGLOBULINS FROM BOVINE WHEY

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It has already been reported that the principal protein of bovine colostrum is immune lactoglobulin, and a procedure has been described for the isolation of this protein (1). While the immune properties of colostrum have been generally recognized, it is widely believed that this is a specific quality of colostrum, and that immune properties are absent from the later milk or are present only in traces. In this communication, it will be demonstrated that the immune proteins are present in normal milk as shown by an electrophoretic analysis of the whey and by the isolation of these proteins in electrophoretically homogeneous state. The immune activity of milk like that of colostrum is associated with two different immune lactoglobulins which differ in electrophoretic mobility, solubility, and analytical composition (1-3). For convenience, they may be described as euglobulin and pseudoglobulin. Some of the properties of these proteins will be discussed.

EXPERIMENTAL

Electrophoretic Analysis of Whey—These studies were performed at 1° in a Tiselius apparatus equipped with the Longworth schlieren scanning device. Unless otherwise specified, the analyses were made with a veronal (diethyl barbiturate) buffer at pH 8.4 to 8.6 and at an ionic strength of 0.1. Only the descending boundaries were observed.

The fat was removed from the whole milk by centrifuging in a Dé Laval cream separator two or three times. The skim milk was slowly acidified with 0.1 M HCl to pH 4.6 ± 0.1 , and the casein removed by filtration. Because of the low protein content of the whey, some concentration was necessary to obtain satisfactory electrophoretic analyses. The whey was therefore cautiously neutralized with 0.1 M NaOH, filtered clear, dialyzed against distilled water to lower the content of diffusible ions and lactose, and then dried from the frozen state under a high vacuum. The dried whey proteins prepared by this method showed no detectable changes in the mobility or relative concentrations of the various components.

Table I presents the data obtained on the electrophoresis of a whey sample from normal milk performed at three different protein concentra-

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tions. The electrophoretic pattern obtained at 1.85 per cent is shown in Fig. 1. The two slowest moving components are the immune lactoglobulin components whose isolation is described below. In this particular preparation the two components are not distinct from each other. Better resolu-

TABLE I

Electrophoretic Analyses of Bovine Whey at Different Protein Concentrations

These analyses were performed at 1° in a veronal buffer at pH 8.6 and at an ionic strength of 0.1. All of the samples were dialyzed and were concentrated by drying from the frozen state prior to equilibration with the buffer solution used in the electrophoretic analysis. Mobilities were calculated from descending patterns after electrophoresis for 250 minutes. Mobilities are in sq. cm. per volt per second $\times 10^{-5}$.

Concentration	Immune lactoglobulin (euglobulin)		Immune lactoglobulin (pseudoglobulin)		Component		Component		β -Lactoglobulin		Component	
	Concentration	u	Concentration	u	Concentration	u	Concentration	u	Concentration	u	Concentration	u
per cent	per cent		per cent		per cent		per cent		per cent		per cent	
0.51	5	-1.7	5	-2.1	23	-3.7	14	-4.7	48	-5.6	5	-6.7
1.23	6	-1.7	4	-2.5	18	-3.6	12	-4.5	55	-5.1	5	-6.4
1.85	6	-1.5	4	-2.4	16	-3.2	9	-4.1	58	-5.0	7	-6.1

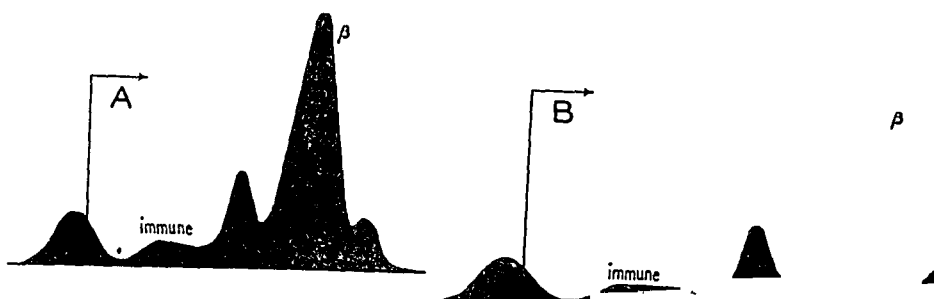


FIG. 1. Electrophoretic patterns of normal whey from photographs taken at (A) 166 and (B) 250 minutes. The protein concentration was 1.85 per cent. The data for this experiment are given in Table I.

tion has been obtained with other samples of whey, particularly those from hyperimmune animals.

The data in Table I indicate that the apparent amount and the mobility of some of the boundaries, particularly β -lactoglobulin, are influenced by the protein concentration at which the electrophoresis is performed. In whey, the component with mobility -4.1 to -4.7×10^{-5} sq. cm. per volt per second can be detected only as an asymmetry on the β -lactoglobulin pattern after prolonged electrophoresis.

Electrophoresis of Hyperimmune Whey—The immunization of these animals has been studied by Dr. A. Holm of these laboratories and the work will be reported by him elsewhere. Studies performed on the immune globulins of the colostrum and plasma from these animals have already been presented (1-3).

The whey obtained from hyperimmune animals does not differ in composition from that of normal animals except that the immune components may be greatly increased in amount. In Fig. 2 are shown the patterns obtained with the whey from two hyperimmune cows. For the whey shown in Fig. 2, A, there were 32 per cent of the immune protein and only

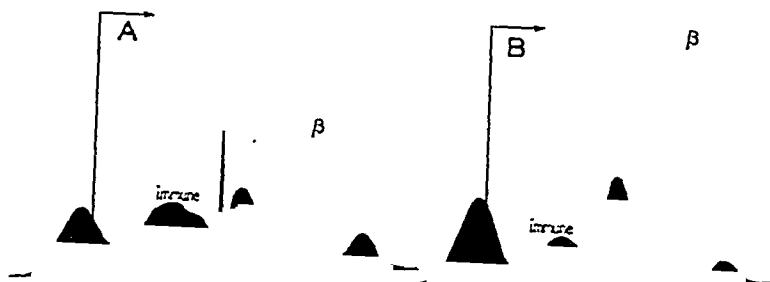


FIG. 2. Electrophoretic patterns of whey obtained from two hyperimmune cows. A contained 31 per cent of the immune components, and B only 14 per cent. The photographs were taken at 225 minutes (A), and 200 minutes (B). The pH was 8.4 and the protein concentration 2.6 per cent for both runs.

45 per cent β -lactoglobulin, while in Fig. 2, B, there were 14 per cent of the immune protein and 64 per cent of the β -lactoglobulin. Because of the high protein concentration employed (2.6 per cent), the β -lactoglobulin appeared to be a single boundary in both experiments. The sample in Fig. 2, A showed the most extreme change in composition from normal whey for any of the preparations which were investigated. The anomaly in the pattern of Fig. 2, A, has been observed occasionally with samples of whey.

In Table II are presented the data obtained with the whey during the course of immunization. No attempt was made in these runs to evaluate the asymmetry of the β -lactoglobulin boundary, and this has been considered as due to one component. Likewise, the two immune components have been considered together, and the mobility is for the peak position of the total immune protein.

The relative concentration of the immune components in the whey of Cow 1 showed no significant trend during the course of immunization.

However, in Cow 2 the relative concentration of these proteins increased from 10 to 32 per cent. Cow 3 was dry at the time that hyperimmunization was begun and no control data could be obtained on the whey of this animal.

At various times, whey samples have shown boundaries which have migrated more rapidly than the component at -5.3 to -5.8×10^{-5} sq. cm. per volt per second. While such components appear in Table II only for the whey of the unimmunized animals, they have also been ob-

TABLE II
Electrophoretic Analyses of Bovine Whey

These analyses were performed at 1° in veronal buffer at pH 8.4 to 8.6 and at an ionic strength of 0.1. All of the samples were dialyzed and were concentrated by drying from the frozen state prior to equilibration with the buffer used in the analysis. Mobilities were calculated from descending boundaries after electrophoresis for 200 to 250 minutes. Mobilities are in sq. cm. per volt per second $\times 10^{-5}$. The concentration given is for both immune components, and the mobility is for the peak position of the unresolved euglobulin and pseudoglobulin.

Cow No.	Time of immunization	Concentration	Immune components		Component		β -Lactoglobulin		Component		Component	
			Concentration	u	Concentration	u	Concentration	u	Concentration	u	Concentration	u
	days	per cent	per cent		per cent		per cent		per cent		per cent	
1	0	2.7	17	-1.8	12	-3.0	60	-4.2	6	-5.7	5	-7.5
	28*	2.6	14	-1.7	18	-3.0	64	-3.9	4	-5.3		
	42	1.6	17	-1.9	21	-3.2	58	-4.2	4	-5.5		
	56	1.3	19	-1.8	21	-3.2	56	-4.1	4	-5.5		
2	0	3.0	10	-2.0	14	-3.0	67	-4.0	4	-5.8	5	-6.8
	28	1.8	22	-1.9	22	-3.1	52	-4.0	4	-5.5		
	42	2.4	24	-1.6	20	-3.1	49	-4.2	7	-5.2		
	56*	2.6	32	-1.5	15	-2.7	48	-3.9	5	-5.1		
3	56	2.0	15	-2.0	24	-3.0	50	-4.1	11	-5.5		

* These samples are shown in Fig. 2.

served in the hyperimmune whey. We have no explanation to offer at the present time for the irregular appearance of these boundaries.

Although whey apparently exhibits only a few boundaries in its electrophoretic pattern, this fluid is extremely complex and some of the electrophoretic components are composite in nature. When a series of whey fractions is prepared by precipitation with ammonium sulfate, or with ethanol at low temperatures, made in short steps of concentration increments, new components are observed in the patterns of some of the fractions.

Isolation of Immune Components—These components were obtained in electrophoretically homogeneous state by a fractionation procedure in

which ammonium sulfate was used. The fat and casein were removed from the whole milk of hyperimmune cows as described above in the preparation of samples for electrophoretic analysis. The whey was then adjusted to pH 6.5 with 0.1 M NaOH, and solid ammonium sulfate added to 0.5 saturation to precipitate the crude globulin (Fraction A). The precipitate Fraction B was collected from the filtrate of Fraction A at complete saturation with ammonium sulfate. Fraction A was redissolved at about 3 per cent protein concentration, the pH was brought to 4.6 ± 0.1 , and ammonium sulfate added to 0.25 saturation. The precipitate (Fraction C) was removed by centrifuging. The supernatant was filtered through a thick layer of paper pulp and then through a sterilizing pad. The immune proteins (Fraction D) were precipitated from the supernatant at 0.4 saturation with ammonium sulfate and at pH 6.0.

Fraction D, which contained 80 per cent of the immune proteins, was reworked by dissolving in water at 1°, adjusting to pH 4.5, and removing the insoluble residue by filtration. Fraction E was precipitated at 0.3 saturation with ammonium sulfate. The supernatant was brought to pH 6.0 and 0.4 saturation with ammonium sulfate and the precipitate Fraction F was collected. Fractions E and F were dialyzed separately against distilled water at 2°. Both precipitates were resolved into water-soluble (pseudoglobulin) and water-insoluble (euglobulin) fractions; they were collected separately, and each portion was analyzed electrophoretically. The data for the entire fractionation are given in Table III.

It is apparent from the data in Table III that both the pseudoglobulin fractions (E and F) appear to be homogeneous, while only the euglobulin Fraction F is homogeneous. However, the pseudoglobulin Fraction E apparently contains some of the euglobulin, as is shown by its mobility which is intermediate between those of the more homogeneous Fraction F preparations.

The representative fractionation described above was performed with 86 liters of whey which contained 0.57 per cent protein. Since the immune lactoglobulin was 13 per cent of the whey protein as estimated from the electrophoretic pattern, the total amount originally present was 63.7 gm. The yield of the partially purified precipitate Fraction D was 54 gm., which represented approximately 68 per cent of the immune protein present in the whey. The yield of the pseudoglobulin Fraction F was 5 gm. and euglobulin Fraction F 9 gm. Both globulins possessed immune activity for the antigens which were used in the hyperimmunization.

While the fractionation presented above is described for the whey from hyperimmune cows, it is equally effective for normal whey. The example was deliberately chosen for presentation because of its resemblance to normal whey in the low content of immune protein.

The successful isolation of these proteins has depended in large part on the elimination of the components which migrate in the range of -3.0 to -3.7×10^{-5} sq. cm. per volt per second. Sørensen and Sørensen (4) have commented on the fact that the whey fraction precipitated at low sulfate concentrations "is characterized by such a slimy, sticky consistency that it is very difficult to reprecipitate and purify, and even so, to fractionate." We have found that this is entirely due to euglobulin-like pro-

TABLE III

Electrophoretic Analyses of Precipitates from Bovine Whey Obtained by Ammonium Sulfate Precipitation

Electrophoresis was performed at 1° in veronal buffer at pH 8.6 and at an ionic strength of 0.1. Mobilities are in sq. cm. per volt per second $\times 10^{-5}$. Values given in parentheses under each fraction refer to limits of saturation of ammonium sulfate. The vertical columns do not always include the same component, with the exception of the immune lactoglobulin.

Fraction	Immune lactoglobulin		Component 2		Component 3		Component 4		Component 5	
	Concentration	μ	Concentration	μ	Concentration	μ	Concentration	μ	Concentration	μ
	per cent		per cent		per cent		per cent		per cent	
Whey.....	13	-1.8	22	-3.7	59	-4.8	6	-5.7		
A (0-0.5).....	37	-2.0	32	-3.3	8	-4.2	23	-5.0		
B (0.5-1.0).....	3	-2.3	19	-3.1	66	-4.2	6	-5.9	6	-7.0
C (0-0.25).....	21	-2.2	46	-4.1	30	-5.2			3	-7.2
D (0.25-0.4).....	80	-2.1	20	-3.7						
E (0-0.3), euglobulin.....	30	-2.0	66	-3.0	4	-4.8				
" (0-0.3), pseudo-globulin.....	100	-2.2								
F (0.3-0.4), euglobulin.....	100	-1.8								
F (0.3-0.4) pseudo-globulin.....	100	-2.5								

tein which migrates in the range of -3.0 to -3.7×10^{-5} sq. cm. per volt per second. These components, which migrate with the same mobility as the β -globulins of serum, also resemble the β -globulins in their solubility properties and in the occasional presence of an anomaly on the electrophoretic pattern (Fig. 2, A). Lyophilized powders rich in this material do not readily redissolve in salt solutions at pH 7.0 to 7.5 and the solutions are quite turbid. Refractionation of this material shows it to be complex in character, yielding many distinct electrophoretic boundaries. In contrast to the crude fractions, the more homogeneous immune lactoglobulins are freely soluble in neutral saline, and the lyophilized powders dissolve to form clear and colorless solutions.

It should be emphasized that the classical methods of preparing lactoglobulin (5-7) by repeated precipitation with half saturated ammonium sulfate, or with saturated magnesium sulfate, yield preparations which show complex electrophoretic patterns. For example a whey protein preparation twice precipitated at 0.4 saturation with ammonium sulfate

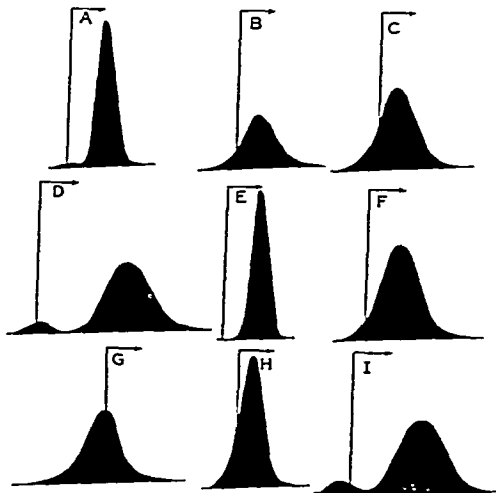


FIG. 3. Descending patterns obtained on electrophoresis of the purified immune lactoglobulins. The pseudoglobulin is shown in *A*, acetate buffer at pH 3.90, at a protein concentration of 1.3 per cent after 150 minutes; *B*, acetate, pH 5.11, concentration 0.8 per cent, 250 minutes; *C*, cacodylate, pH 6.81, concentration 1.1 per cent, 250 minutes; and *D*, veronal, pH 8.55, concentration 1.6 per cent, 250 minutes. The euglobulin is shown in *E*, acetate, pH 3.81, concentration 1.2 per cent, 140 minutes; *F*, acetate, pH 5.12, concentration 1.1 per cent, 250 minutes; *G*, cacodylate, pH 6.13, concentration 1.0 per cent, 250 minutes; *H*, cacodylate, pH 6.82, concentration 1.3 per cent, 250 minutes; and *I*, veronal, pH 8.65, concentration 1.4 per cent, 250 minutes.

contained about 40 per cent of the immune lactoglobulins, and showed four distinct boundaries.

Electrophoretic Homogeneity—The purified euglobulin Fraction F and pseudoglobulin Fraction F were studied electrophoretically in the Tiselius apparatus with univalent buffers at an ionic strength of 0.1. Both of these proteins migrated as single components at all pH values at which observations were made. Some of the electrophoretic patterns which were obtained are shown in Fig. 3. These proteins gave somewhat sharper patterns and less symmetrical spreading than was found earlier for the immune proteins from bovine colostrum (1).

Isoelectric Points—In Fig. 4 the electrophoretic mobilities are shown as a function of the pH for both of the immune lactoglobulins as obtained from descending migrations in univalent buffers at an ionic strength of 0.1. The isoelectric point of the pseudoglobulin is at pH 5.6 and of the euglobulin at pH 6.05. These values are in the pH range previously observed with the immune proteins from colostrum (1).

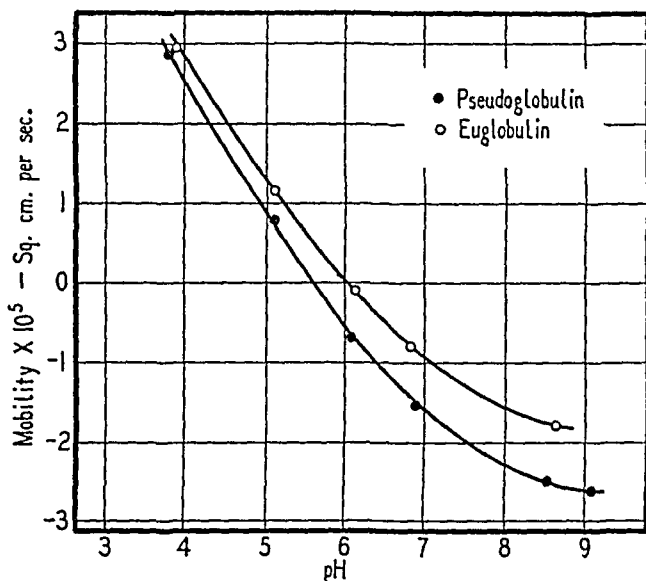


FIG. 4. Electrophoretic mobility as a function of pH for the euglobulin and pseudoglobulin of bovine whey. All of the measurements were determined from descending migrations at 1° in univalent buffers at an ionic strength of 0.1. The pH values were determined with a glass electrode at 25°.

Analytical Composition—Some data for the homogeneous preparations are presented in Table IV. The results are calculated on the ash-free, dry basis; the ash content is on the dry basis. Mr. J. F. Alicino of The Squibb Institute for Medical Research performed the elementary analyses. No phosphorus could be detected in these proteins. The hexose determinations were made by the orcinol method with glucose as a standard (8). Hexosamine was determined by the method of Palmer, Smyth, and Meyer (9), with a Beckman spectrophotometer to measure the photometric densities of 540 mμ.

Absorption Spectra—The ultraviolet spectra of the homogeneous lactoglobulins are shown in Fig. 5. The measurements were made at room temperature with a Beckman spectrophotometer with a hydrogen lamp as the light source. The proteins were dissolved in 0.15 M sodium chloride,

and adjusted with 0.1 M sodium hydroxide to pH 7.0 ± 0.2 . The two preparations differ only slightly and closely resemble the immune lacto-

TABLE IV
Some Analytical Data on Immune Lactoglobulins of Milk

Constituent	Pseudoglobulin	Euglobulin
	<i>per cent</i>	<i>per cent</i>
C.....	51.94	53.95
H.....	6.96	7.12
N, Dumas.....	15.29	16.05
S.....	1.00	1.01
Ash.....	0.45	>0.05
Hexose.....	2.49	2.94
Hexosamine.....	1.27	1.32

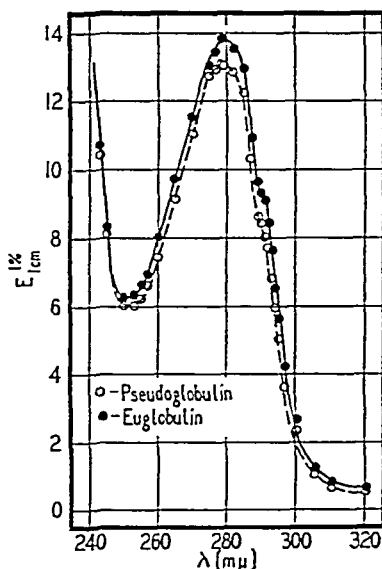


FIG. 5. Ultraviolet absorption spectra of the immune lactoglobulin of bovine whey globulins from colostrum (Smith and Coy (3)). We are indebted to Dr. N. H. Coy for these measurements.

Diffusion Constants—These were measured in the electrophoresis cell by the method of Longworth (10) from photographs taken by the schlieren scanning method. The constants were calculated from the formula $D = A^2/4\pi H^2t$ where A is the diffusion area in sq. cm., H the maximum height

of the curve in cm., t the time in seconds, and D the diffusion constant in sq. cm. per second.

The runs were performed at 1.5° in veronal buffer of 0.1 ionic strength at pH 8.6. From five to seven photographs were taken at intervals from about 14 to 70 hours and the values averaged. Each run was performed in duplicate, both halves of the cell being used separately. It was found for the pseudoglobulin, at a concentration of 1 per cent, that $D_{1.5,0} = 1.86$ and 1.90×10^{-7} , from which the calculated values of $D_{20,w} = 3.48$ and 3.55×10^{-7} sq. cm. per second. For the euglobulin, at 1.2 per cent, $D_{1.5,0} = 1.66$ and 1.81×10^{-7} were obtained, and the calculated values $D_{20,w} = 3.10$ and 3.38×10^{-7} sq. cm. per second. These values are within the range previously found for bovine colostrum and plasma immune globulins (1).

Studies in the Ultracentrifuge—Dr. J. L. Oncley of the Department of Physical Chemistry, Harvard Medical School, has examined some of our preparations of the bovine immune proteins. We are greatly indebted to Dr. Oncley for putting these results at our disposal.

The air-driven ultracentrifuge designed by Bauer and Pickels was used in these studies. Analyses were performed with 0.15 M sodium chloride as solvent. The milk preparations were those described in this paper. The colostrum and plasma proteins were the same as those discussed earlier (1). The results are given in Table V.

All of the preparations contained two or three sedimenting components, and in that respect resemble the human γ -globulin preparations studied by Williams and his collaborators (11). However, only the euglobulin preparations contained any heavy material with sedimentation constants (S_{20}) in the range of 18 to 20 Svedberg units.

The regular occurrence of a component with S_{20} about 10 Svedberg units in association with a principal component of S_{20} of about 7 Svedberg units suggests a close relationship between them. It does not seem likely that the same impurity would be present in globulin preparations from colostrum, milk, and plasma, and in each case possess the same electrophoretic mobility at various pH values as each of the different proteins involved. It is possible that the three components have a polymeric relationship to one another. We have no very good data for the estimation of f/f_0 values for these components, but the molecules with $S_{20} = 10$ Svedberg units might be approximately twice the size of those with $S_{20} = 7$ units; those with $S_{20} = 18$ to 20 units might be about 6 to 8 times the size of those with $S_{20} = 7$ units. It should be recalled that the antipneumococcus serum globulin from the cow was found to possess a sedimentation constant of 18.1 Svedberg units and a molecular weight of 910,000 (12).

In a study of the whey proteins performed with the ultracentrifuge, Pedersen (13) found three main boundaries: an α component with $S_{20} = 1.8$

Svedberg units (Kekwick's lactalbumin), a β component with $S_{20} = 3.0$ units (Palmer's lactoglobulin), and a γ component with $S_{20} = 7.0$ to 7.4 units. The last component, which represented about 10 per cent of the whey protein, was tentatively identified by Pedersen as the classical lactoglobulin. The sedimentation constant of this protein in whey is in agreement with that found for the lactoglobulins isolated from milk and colostrum. The results of the electrophoretic and ultracentrifugal analyses also agree in the regular occurrence of the immune lactoglobulins to the extent of about 10 per cent in normal whey.

The sedimentation constant of the principal component of the colostrum and milk proteins, like that of the γ - and the T-globulins, was about 7

TABLE V

Composition of Bovine Immune Proteins in Ultracentrifuge

Each component is identified by the approximate value of the sedimentation constant given in Svedberg units.

Protein	$S_{20} = 7$ units	$S_{20} = 10$ units	$S_{20} = 20$ units
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Milk pseudoglobulin.....	90	10	0
Colostrum pseudoglobulin*.....	88	6	0
Milk euglobulin.....	76	12	12
Colostrum euglobulin.....	85	5	10
Plasma T-globulin.....	88	12	0
“ γ -globulin A.....	90	10	0
“ “ B.....	92	8	0

* About 6 per cent of this protein sedimented with a value of $S_{20} = 2$ to 3 Svedberg units.

Svedberg units. The diffusion constants estimated for the different preparations are values for the total protein. The high content of the fast moving component ($S_{20} = 18$ to 20 units) in the two euglobulins is reflected in their lower diffusion constants: $D_{10,20} = 3.24$ and 3.34×10^{-7} sq. cm. per second for the milk and colostrum preparations. The diffusion constant for the colostrum pseudoglobulin, which contained 88 per cent of one component, was 3.86×10^{-7} sq. cm. per second (1). The true diffusion constant of the homogeneous lactoglobulin must therefore be slightly greater than 3.9×10^{-7} sq. cm. per second. Using this value together with $S_{20} = 7$ Svedberg units and $V = 0.75$ (assumed) in the Svedberg formula yields a molecular weight of 180,000.

DISCUSSION

The high levels of immunity generally present in colostrum have served to obscure the fact that immunity is also present in the later milk. Though

present in small amount, the immune proteins occur regularly in the whey of normal animals, as is shown by electrophoretic analysis and by the ultracentrifuge studies of Pedersen.

The immune globulins of the colostrum and milk are probably identical. However, this is not easily proved because of the variable amounts of the two immune proteins which are present and their similarity in properties which make it an extremely difficult matter to be certain of their complete separation. A further difficulty is the lack of homogeneity in the ultracentrifuge. Moreover, preliminary observations, as yet unpublished, show that the lactoglobulins give solubility data characteristic of preparations which contain more than a single component in the solid phase.

The author acknowledges the technical assistance of Leo Zuckerman and Douglas M. Brown.

SUMMARY

1. Electrophoretic analysis has shown that the immune lactoglobulins constitute about 10 per cent of the protein in normal bovine whey. During hyperimmunization the immune components may increase considerably, although this does not occur regularly.

2. A method has been described for the isolation from whey of the euglobulin and pseudoglobulin in electrophoretically homogeneous form. Immune activity is associated with both of these proteins.

3. The isolated proteins have been studied in the Tiselius apparatus at different pH values, and the proteins have been characterized by their isoelectric points, diffusion constants, absorption spectra, and other properties.

4. Studies in the ultracentrifuge reveal that all of the isolated bovine immune proteins contain more than one component. The principal component (76 to 92 per cent) has a molecular weight of about 180,000.

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STEROIDS

VII. THE PREPARATION OF ANDROSTAN-3(β)-OL-7-ONE FROM DEHYDROISOANDROSTERONE*

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Seven years ago the authors (2) reported the isolation from equine pregnancy urine of a new hydroxy ketone, m.p. 187–187.5° (uncorrected), $[\alpha]_D = -160^\circ$ (dioxane), m.p. of benzoate, 206–208° (uncorrected), the structure of which was proved to be androstan-3(β)-ol-*x*-one, an isomer of androsterone differing in the spatial orientation of the 3-hydroxyl group and in the position of the ketonic oxygen atom. More recently, Oppenauer (3) has obtained from the same source a similar digitonin-precipitable hydroxy ketone, $C_{19}H_{30}O_2$, m.p. 190.5–191° (corrected), $[\alpha]_D = -157^\circ$ (dioxane), m.p. of benzoate, 209–211° (corrected); while the two products have not been directly compared, there can be no reasonable doubt of their identity.

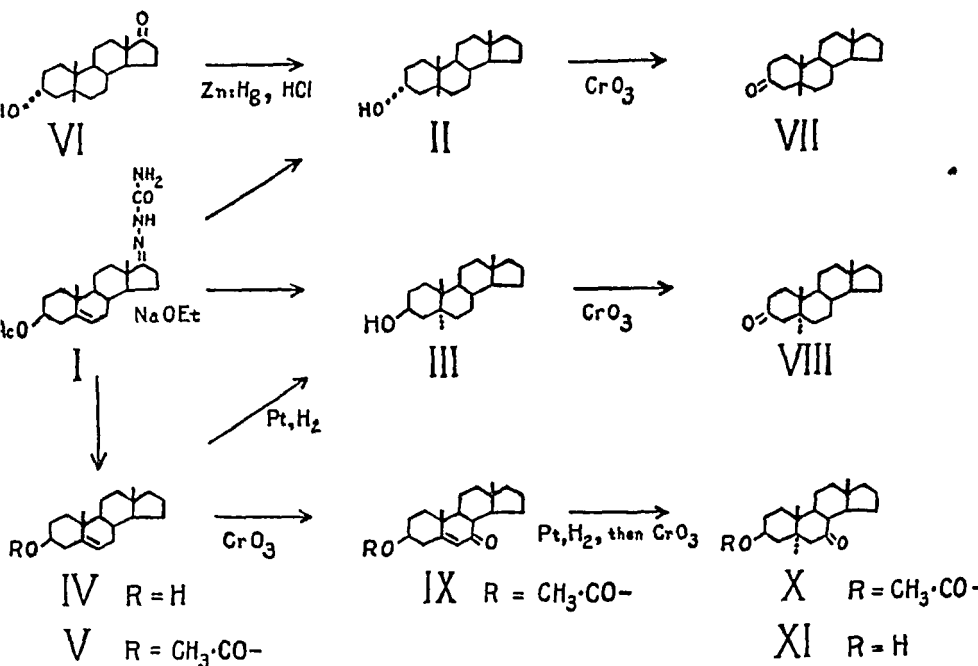
In the original investigation, the location of the carbonyl group was not established. With the object of elucidating this last undetermined point of structure, a survey was conducted of the effect on optical rotatory power of the substitution of a ketonic oxygen atom in the various positions in the steroid ring skeleton, the results¹ of which show that the contributions to the molar rotation ($[M]_D = [\alpha]_D \times \text{mol. wt.}/100$) of a 6-, 7-, 11-, and 12-keto group are respectively and approximately -200° to -400° , -230° to -320° , $+260^\circ$, and $+210^\circ$ to $+380^\circ$. The observed difference (-480°) between the molar rotation of the urinary androstan-3(β)-ol-*x*-one ($[M]_D = -464^\circ$) and of androstan-3(β)-ol ($[M]_D = +16^\circ$) suggested the presence of the carbonyl group in the 6 or 7 position. Accordingly, androstan-3(β)-ol-7-one (XI) has been prepared from dehydroisoandrosterone as described below. While the rotation of XI is in accordance with expectation ($[M]_D = -200^\circ$), the compound (m.p. 130–131°) is not identical with the urinary metabolite.

The Wolff-Kishner reduction of dehydroisoandrosterone acetate semicarbazone (I) with sodium ethylate at 180° led to a difficultly separable mixture of Δ^5 -androsten-3(β)-ol (IV), androstan-3(β)-ol (III), and etiocholan-3(α)-ol (II). Although the crude reaction mixture readily crystallized

* Communicated to the American Society of Biological Chemists, 1941 (1).

¹ Fuller details will be published in a later communication.

in long needles (m.p. 120–130°) with the appearance of homogeneity, fractionation with digitonin was found requisite to the isolation of the constituents. The digitonin-precipitable β portion (approximately 60 per cent) was comprised principally of a fraction sublimable *in vacuo* between 105–115°, which gave, on one recrystallization, the main product in the form of slender needles melting sharply at 133–134° and exhibiting $[\alpha]_D = -65.3^\circ$ in dioxane (40 per cent yield from I). The latter proved to be mixed crystals of IV and III in the approximate molar proportion of 2:1 respectively.



Bromine and hydrogen titration indicated 0.70 and 0.77 ethenoid linkage. The hydrogenation product, $\text{C}_{19}\text{H}_{32}\text{O}$, formed in quantitative yield, had a melting point of 148–149°, $[\alpha]_D = +5.9^\circ \pm 7^\circ$ (dioxane) in agreement with androstan-3(β)-ol (III, m.p. 147.5–148°, $[\alpha]_D = +0.9^\circ$ in chloroform) obtained by Prelog, Ruzicka, and Wieland (4) by saturation of Δ^5 -androsten-3(β)-ol. Resolution of the coordination complex was achieved by application of the principle of Schoenheimer (5) that Δ^5 -unsaturated 3(β)-hydroxy steroids, as their dibromides, are not precipitated with digitonin. Bromination of the mixed crystals gave the dibromide of IV, m.p. 110°, which, on dehalogenation, yielded Δ^5 -androsten-3(β)-ol (IV), m.p. 135–136.5°, $[\alpha]_D = -68^\circ$ (dioxane). Treatment of the mother liquor from the bromo derivative with digitonin, and dissociation of the insoluble addition product, led to androstan-3(β)-ol (III), identical with the product of hydro-

genation of the coordination complex. The α fraction (about 40 per cent) consisted for the most part of etiocholan-3(α)-ol (II), obtained either by sublimation and repeated recrystallization to a constant melting point of 143–144°, or by chromatographic separation, and identified by comparison with II obtained by the Clemmensen reduction of etiocholan-3(α)-ol-17-one (VI). For purposes of characterization, small quantities of androstan-3-one (VIII) and of etiocholan-3-one (VII) were prepared by the chromic acid oxidation of III and II respectively.

Saturation of the double bond and epimerization of the hydroxyl group in the course of the Wolff-Kishner reduction of a Δ^5 -unsaturated 3(β)-hydroxy steroid have previously been observed. Dutcher and Wintersteiner (6) obtained from Δ^4 -cholesten-3-one, the normal product, Δ^4 -cholestene (40 per cent), and also cholestan-3(β)-ol and coprostan-3(α)-ol (35 per cent). In both their investigation and ours, the only saturated products obtained are those in which the substituent at C₃ and that at the newly created center of asymmetry at C₅ are trans oriented each with respect to the other, which keeps with the finding of Windaus (7) that, on epimerization of the saturated 3-hydroxy steroids with sodium ethylate at 180°, the 3,5 transoid form predominates over the cisoid in the ratio of about 9:1.

Since the completion of this investigation, Butenandt and Suranyi (8) have described the Wolff-Kishner reduction of dehydroisoandrosterone at 145° to a product, readily separable in high yield by direct crystallization, which melted at 131° (corrected?) and exhibited $[\alpha]_D^{20} = -48^\circ$ (ethanol), and to which structure IV is assigned. Whether this material represents the coordination complex of IV and III characterized above, or IV in pure state, is not apparent, as the degree of unsaturation is not recorded by the German workers. Neither is it certain that the purest Δ^5 -androsten-3(β)-ol ($[\alpha]_D = -68^\circ$) obtained by us on debromination of the dibromide is uncontaminated with the saturated androstan-3(β)-ol. As the complex ($[\alpha]_D = -65^\circ$) is made up of approximately 33 per cent of III, which is practically inert optically (below and (4)), IV in pure form should be levorotatory to the extent of about +100°. While the findings of Butenandt and Suranyi (8) and ourselves agree in the main, they are quite irreconcilable with those of Raoul and Meunier (9) who describe Δ^5 -androsten-3(β)-ol, also from the Wolff-Kishner reduction of I, as melting at 104° and showing $[\alpha]_D = +18.5^\circ$. No evidence of unsaturation is presented, nor are the observed carbon and hydrogen values in agreement with the theory for IV. Moreover, as androstan-3(β)-ol has practically zero rotation (below and (4)), and the contribution to the molar rotation of a 5,6-ethylenic linkage is known to be -200 to -400° (Callow and Young (10)), the specific rotation of IV may be calculated to lie in the range -55° to -110°.

Acetylation of the coordination complex and repeated recrystallization of the products yielded the unsaturated acetate V, melting at 93–94°, which agrees with the value (91–93°) recorded by Butenandt and Suranyi (8). Treatment of this with chromic anhydride at 55° introduced a carbonyl oxygen atom in conjugation with the double bond (compound IX), as evidenced by the ultraviolet absorption spectrum (Fig. 1), which is characteristic of that of a ketone in conjugation with an exocyclic double bond (see Woodward (11)). Catalytic hydrogenation of IX followed by

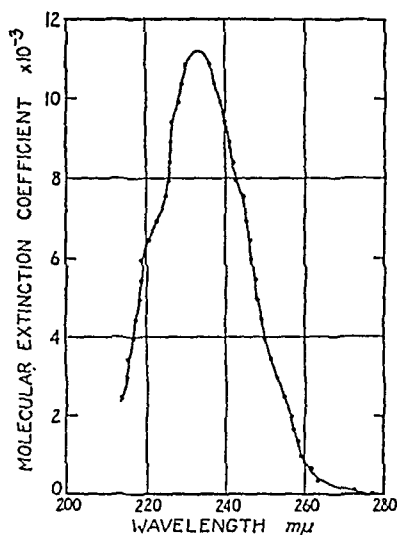


FIG. 1. Absorption spectrum of Δ^5 -androsten-3(β)-ol-7-one acetate (IX) in ethanol

back oxidation of the epimeric mixture of 7-(α and β)-hydroxyandrostan-3(β)-ol 3-acetate gave X, m.p. 110–113°, not identical with the acetate (m.p. 105–107°) of the urinary androstan-3(β)-ol- x -one.

EXPERIMENTAL

Melting points were taken with the Kofler-Hilbeck micro apparatus; the recorded values are corrected. We are much indebted to Mrs. A. C. Jewitt for all microanalyses.

Reduction (Wolff-Kishner) of Dehydroisoandrosterone Acetate Semicarbazone (I)—To 2.01 gm. of I was added sodium ethylate solution prepared from 5.0 gm. of sodium and 65 ml. of absolute ethanol, and the mixture was heated in a sealed tube for 10 hours at 180°. After dilution to 10 volumes, the products were extracted with ether (three portions of 75 ml.). The combined ethereal extracts were washed with water (3 \times 50 ml.) and distilled to dryness to give 1.6 gm. of light yellow oil. While the latter readily crystallized from benzene-ligroin in well defined needles which softened at

116° and melted between 120–130°, no worth while degree of purification could be effected by repeated treatment in this manner or by sublimation *in vacuo*. Accordingly, the mixture was separated into α and β fractions with digitonin. The oil (1.6 gm.) was taken up in a 1 per cent alcoholic solution of digitonin (containing 5.0 gm.), and the digitonides that were precipitated after 24 hours were collected by filtration and washed with cold ethanol and ether. The combined mother liquor and washings were distilled to 5 ml., and treated with 100 ml. of ether. The precipitated excess digitonin was removed and washed with ether. Evaporation of the filtrate and ether washings gave 0.672 gm. of digitonin-non-precipitable oil (α fraction). The insoluble digitonides, after desiccation, were dissolved in pyridine (30 ml., freshly distilled from anhydrous barium oxide), warmed on the bath for 30 minutes, and poured into 400 ml. of dry ether. After collection and washing of the precipitated digitonin with ether, the combined ethereal solutions were extracted with 2 N hydrochloric acid (3×100 ml.), washed neutral with water, and distilled to yield a semicrystalline β fraction. A second decomposition with pyridine of the reclaimed digitonin gave a slight additional quantity of ether-soluble material (total yield, 0.834 gm.). In several runs, it was found that the number of decompositions requisite to quantitative recovery of the 3(β)-hydroxy steroids from their digitonides varied greatly with the degree of hydration of the solvents employed; with pyridine freshly distilled from anhydrous barium oxide, two treatments sufficed, but with reagent grade pyridine as many as five were required.

Etiocholan-3(α)-ol (II); From Wolff-Kishner Reduction of I—The α fraction yielded etiocholan-3(α)-ol on distillation at 110–120°, followed by repeated recrystallization from ligroin and aqueous acetone until crystals melting sharply at 143–144° were obtained. More satisfactorily, the compound was separated chromatographically. The digitonin-non-precipitable oil (170 mg.), dissolved in petroleum ether, was absorbed on a column (14.5 \times 1 cm.) of alumina (Merck, standardized according to Brockmann, 7 gm.) which had previously been saturated with the same solvent. Elutions were made as indicated in Table I. Recrystallization of Fractions 16 to 27 from aqueous acetone gave II in the form of long needles melting at 144–145°.

$C_{19}H_{26}O$. Calculated.	C 82.54,	H 11.67,	mol. wt. 276
Found.	" 82.53,	" 11.61,	" " 269 (Rast)
	" 82.81,	" 11.70	

From Clemmensen Reduction of Etiocholan-3(α)-ol-17-one (VI)—To a suspension of 63 mg. of etiocholan-3(α)-ol-17-one (isolated from human urine)² in 10 ml. of concentrated hydrochloric acid were added 2.5 gm. of

² We are greatly indebted to Dr. M. M. Hoffman for this material.

freshly amalgamated zinc wool. After refluxing for $2\frac{3}{4}$ hours, the solution was decanted from the mercury, diluted to 10 volumes, and extracted with ether (3×25 ml.). The combined ethereal extracts were washed with *N* sodium hydroxide solution (3×25 ml.) and water (3×25 ml.), and evaporated to dryness. Crystallization of the residue from ligroin gave etiocholan-3(α)-ol, melting at $143-145^\circ$, both alone and on admixture with the product obtained by Wolff-Kishner reduction.

Etiocholan-3-one (VII)—To 103.7 mg. of II in 2 ml. of 90 per cent acetic acid were added 110 mg. of chromic anhydride in 2 ml. of the same

TABLE I

Chromatographic Separation of α Fraction from Wolff-Kishner Reduction of I

Fraction No.	Eluent (20 ml. portions)	Eluate		Compound isolated
		Yield	Nature or m.p.	
		mg.	$^\circ\text{C}$.	
1-2	Ligroin	0		
3-4	Ligroin-benzene (10:1)	0		
5-8	" (5:1)	17.6	Oil	
9-15	" (5:1)	49.7	102-125	Etiocholan-3(α)-ol
16-21	" (5:1)	64.6	125-144	"
22-27	" (2.5:1)	26.6	142-144	"
28	" (1:1)	1.0	137-144	"

solvent. After 1 hour at 4° , the solution was diluted (15 volumes) and extracted with ether (3×10 ml.). The ethereal extracts were washed with *N* sodium hydroxide solution (3×10 ml.) and water (3×10 ml.), and evaporated to dryness. Sublimation of the crystalline residue *in vacuo* and two recrystallizations from aqueous ethanol gave 61.2 mg. of etiocholan-3-one melting at $55-57^\circ$.

Δ^5 -Androsten-3(β)-ol (IV) and Coordination Complex of IV and Androstan-3(β)-ol (III)—Mixed crystals of III and IV were readily obtained in pure state on crystallization from ligroin of that portion of the total β fraction from the Wolff-Kishner reduction of I which sublimed in a high vacuum at $105-115^\circ$. This represents about 60 per cent of the total digintonin-precipitable material and yields from 1 gm. of I approximately 400 mg. of complex melting sharply at $133-134^\circ$.

$2(\text{C}_{19}\text{H}_{30}\text{O}) \cdot 1(\text{C}_{19}\text{H}_{32}\text{O})$. Calculated. C 82.92, H 11.24, I No. 61.6
 Found. " 83.14, " 11.04, " " 64.5
 " 83.17, " 10.77

$[\alpha]_D^{25} = -65.3^\circ \pm 2^\circ$ (1.24% in dioxane)

For Δ^5 -androsten-3(β)-ol (IV), Butenandt and Suranyi (8) record a melting point of 131° , $[\alpha]_D^{25} = +48^\circ$ (alcohol). The degree of unsaturation was not ascertained.

The iodine number was determined by the Rosenmund-Kuhnhenh method (12). The complex (21.7 mg.), in chloroform (2 ml.), with 5 ml. of the pyridine sulfate dibromide reagent, absorbed, after 10 minutes in the dark, 8.84 mg. of bromine (0.70 ethenoid linkage). Approximately two-thirds unsaturation is also indicated by quantitative hydrogenation (below).

The dibromide of IV was obtained by treating 105 mg. of complex in ethanol (1 ml.) with 0.8 N ethanolic bromine solution until a faint yellow coloration persisted. On concentration to 1.5 ml. at room temperature under a stream of nitrogen, and standing overnight, 47.6 mg. of long needles separated which had a melting point of 110° . The mother liquors subsequently yielded androstan-3(β)-ol (III) (see below). For debromination, the crystals (47.6 mg.), in benzene (1 ml.), were refluxed (2 hours) with potassium iodide (40 mg., added in 2 ml. of absolute ethanol). The diluted solution was extracted with ether, and the ether extracts were washed with 10 per cent sodium hyposulfite solution and water, and taken to dryness. Sublimation of the residue in a high vacuum gave 9.7 mg. of Δ^5 -androsten-3(β)-ol (IV) which melted at 135 – 136.5° and showed $[\alpha]_D = -68.3^\circ \pm 2^\circ$ (0.71 per cent in dioxane). Mixed with the coordination complex (m.p. 133 – 134°) the melting point was 133 – 135° .

The acetate of IV was prepared from the complex (380 mg.) by treatment with 5 ml. of acetic anhydride in the same volume of pyridine for 16 hours at room temperature. The crude product (383 mg., m.p. 87 – 90°), collected in the usual way with ether and washed free of pyridine and acids, was sublimed *in vacuo* at 95° and then crystallized from aqueous acetone to give 292 mg. of colorless platelets which showed a yellow coloration with tetranitromethane in chloroform solution and which melted sharply at 93 – 94° . Butenandt and Suranyi (8) record a melting point of 91 – 93° for Δ^5 -androsten-3(β)-ol acetate. Second grade material, melting at 83 – 86° and undoubtedly contaminated with the acetate of androstan-3(β)-ol (III), was obtained on recrystallization of the mother liquors.

Androstan-3(β)-ol (III)—The saturated monoalcohol III was obtained (a) on hydrogenation of the coordination complex, and (b) was separated (as digitonide) from the products of bromination of the complex.

The complex (26.4 mg.), in glacial acetic acid (2 ml.), was agitated in an atmosphere of hydrogen with 18.7 mg. of previously reduced platinum oxide catalyst. Utilization of hydrogen ceased with an uptake of 0.77 mole. After filtration from the platinum, the product (III) was obtained by extraction with ether and purified by sublimation at 120° *in vacuo* and crystallization from aqueous ethanol (10 mg., m.p. 148 – 149°).

$C_{19}H_{32}O$. Calculated, C 82.54, H 11.67; found, C 82.52, H 11.68
 $[\alpha]_D^{25} = +5.9^\circ \pm 7^\circ$ (0.27% in dioxane)

Prelog, Ruzicka, and Wieland (4) record a melting point of 147.5–148° and $[\alpha]_D^{17} = +0.9^\circ \pm 0.9^\circ$ (chloroform).

The mother liquors from the crystallization of Δ^5 -androsten-3(β)-ol dibromide (above) were treated, in the presence of a slight excess of bromine, with 10 ml. of a 1 per cent ethanolic solution of digitonin. The precipitate after 24 hours was collected, washed with ethanol and ether, dried, and dissociated in pyridine (5 ml.) in the manner previously described. Sublimation at 125° *in vacuo* of the ether-soluble residue gave 20 mg. of androstan-3(β)-ol (m.p. 147–149°), identical by mixture melting point determination with the product obtained by hydrogenation.

$C_{19}H_{32}O$. Calculated, C 82.54, H 11.67; found, C 83.1, H 11.76

Androstan-3-one (VIII)—III (10 mg.) in 2 ml. of acetic acid, was oxidized with chromic anhydride (10 mg.) for 16 hours at 4°, and then processed as described for etiocholan-3-one. Sublimation of the neutral residue at 90° *in vacuo* gave 8 mg. of androstan-3-one (VIII) in the form of needles melting at 102–102.5°.

Δ^5 -*Androsten-3(β)-ol-7-one Acetate* (IX)—V (406 mg.), in 45 ml. of glacial acetic acid, was treated with chromic anhydride (360 mg. in 0.2 ml. of water) at 55° for 3 hours, when the solution was diluted (10 volumes) and extracted with ether (3 \times 100 ml.). The combined ethereal extracts were washed with 2 N hydrochloric acid, N potassium hydroxide solution and water (each 3 \times 75 ml.), and distilled. Crystallization of the residue from acetone-pentane gave rosettes of needles of crude IX (83.4 mg., m.p. 170–174°). The melting point was raised to 173.5–174.5° on recrystallization from ligroin and from ethanol (23.4 mg.).

$C_{19}H_{32}O(OCOCH_3)$. Calculated, C 76.32, H 9.15; found, C 76.90, H 9.28

The absorption spectrum (Fig. 1) exhibited $\epsilon_{\max.} = 11,200$ at 234 m μ .

Androstan-3(β)-ol-7-one Acetate (X)—IX (37.0 mg.), in 2.0 ml. of acetic acid, was shaken in an atmosphere of hydrogen with 10.2 mg. of previously reduced platinum oxide catalyst until absorption of the gas ceased (45 minutes); 2.28 moles were utilized. After removal and washing of the catalyst with acetic acid, the epimeric mixture of the 7-(α and β)-hydroxy compounds contained in the filtrate was oxidized (16 hours at 4°) by the addition of 40 mg. of chromic anhydride in 0.5 ml. of water. The neutral fraction was isolated with ether in the usual way. Crystallization from ligroin yielded 30 mg. of X, melting at 110–113°. Mixed with the acetate of androstan-3(β)-ol-x-one (m.p. 105–107°) from equine pregnancy urine, the melting point was 71–90°.

Androstan-3(β)-ol-7-one (XI)—Saponification of 28 mg. of the acetate X was effected in 2 ml. of 4 per cent ethanolic potassium hydroxide solution (24 hours at room temperature). The product, collected by extraction with ether, was sublimed *in vacuo* at 100–120° to give 25.8 mg. of needles which crystallized from aqueous acetone in transparent leaflets (m.p. 124–126.5°). Resublimation of the latter raised the melting point to 130–131°. The compound is precipitated with digitonin; the Zimmermann (13) reaction is negative.

$C_{18}H_{26}O_2$. Calculated, C 78.56, H 10.41; found, C 78.63, H 10.50
 $[\alpha]_D^{25} = -68.7^\circ \pm 6^\circ$ (0.415% in dioxane)

We wish to thank the Associate Committee on Medical Research of the National Research Council (Ottawa) and Charles E. Frosst and Company, Montreal, for the support of the investigation. One of us (A. F. M.) is much indebted to the Banting Research Foundation for a personal grant.

SUMMARY

The Wolff-Kishner reduction of dehydroisoandrosterone gives Δ^5 -androst-3(β)-ol and androstan-3(β)-ol, which form mixed crystals together, and etiocholan-3(α)-ol. Oxidation of Δ^5 -androst-3(β)-ol with chromic anhydride leads to Δ^5 -androst-3(α)-ol-7-one which is reduced to androstan-3(β)-ol-7-one. The latter is not identical with the androstan-3(β)-ol- α -one excreted during gestation in the mare.

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STEROIDS

VIII. A COLORIMETRIC METHOD FOR THE ESTIMATION OF REDUCING STEROIDS*

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The pioneer investigations of Kendall, Reichstein, Wintersteiner, and collaborators (reviewed by Reichstein and Shoppee (2)) on the structure of the steroids of the adrenal cortex early revealed the strongly reducing sugar-like property of many members of this series (including all of the active hormones) which is imparted by the presence in the side chain of the primary α -ketol grouping characteristic of fructose. To provide a simple chemical method for the estimation of small quantities of reducing steroids of this type, the reduction of phosphomolybdic acid to molybdenum blue has been quantitatively and satisfactorily standardized.

In preliminary qualitative experiments the possible use of 2,6-dichlorophenol indophenol as oxidant was explored. While the dye is decolorized by desoxycorticosterone on being heated (sealed tube) at 100° in an inert atmosphere, the slow rate of reduction and the requisite anaerobic conditions precluded a rapid and simple technique. With phosphomolybdic acid (Folin-Wu reagent (3)) in a medium of acetic acid, reduction takes place rapidly and is not appreciably influenced by atmospheric oxygen. Accordingly the latter reaction was selected for standardization.

Development of the molybdenum blue color (Fig. 1), as measured in the photoelectric colorimeter at 650 to 660 m μ , proceeds rapidly at 100° during the first 30 minutes, after which time the intensity gradient falls off sufficiently to permit reproducible results to be obtained at any arbitrarily chosen time interval thereafter. In practice, a period of heating of 1 hour is allowed for the development of the color. Under these conditions, a series of ten determinations on the same quantity of desoxycorticosterone showed agreement in the optical density ($\log I_0/I$) of the resulting color within ± 2 per cent. Verification of Beer's law that the intensity of the color varies directly with the quantity of reducing substance was then established for desoxycorticosterone, 21-hydroxypregnenolone, and Kendall's Compound E (Fig. 2). These standard curves may thus be applied to estimation of any reducing steroid or suitable biological extract in terms of the reducing equivalent of whichever pure compound as may be selected as standard of reference.

* Preliminary accounts have previously appeared (1).

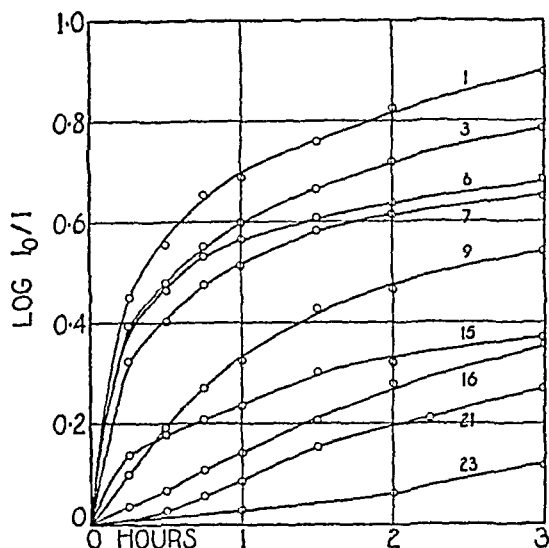


FIG. 1. Rates of reduction of phosphomolybdic acid by 0.303 mm of α -ketolic steroids. The curve numbers correspond to the compound numbers listed in Table I.

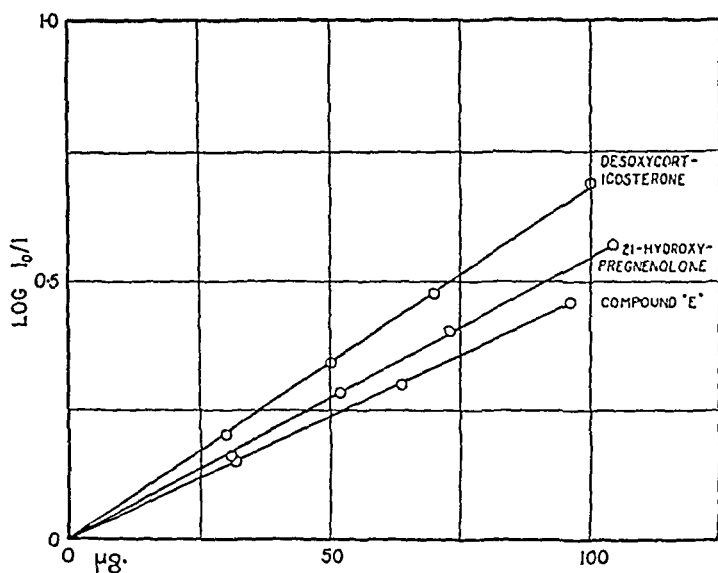


FIG. 2. Relation in intensity of molybdenum blue color to quantity of reducing steroid.

From a survey of the reducing characteristics of a large number of steroids (Figs. 1, 3, and 4; Table I) the following general conclusions have been reached concerning the relation between chemical structure and power to

cause reduction of phosphomolybdic acid. The reference numbers to the various compounds are those assigned in Table I.

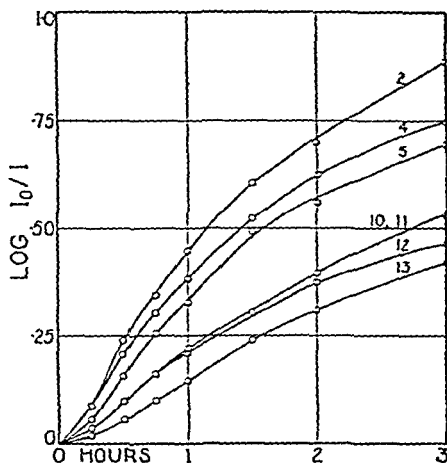


FIG. 3. Rates of reduction of phosphomolybdic acid by 0.303 mM of the acetates of α -ketolic steroids. The curve numbers correspond to the compound numbers listed in Table I.

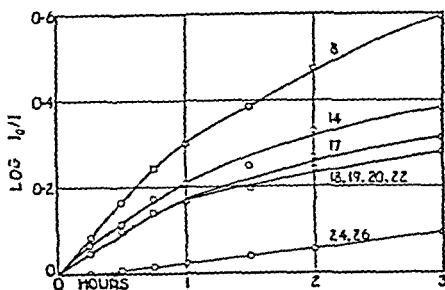
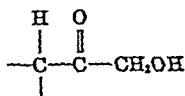


FIG. 4. Rates of reduction of phosphomolybdic acid by 0.303 mM of α,β -unsaturated 3-keto steroids. The curve numbers correspond to the compound numbers listed in Table I.

Group A—The aliphatic primary α -ketol grouping



as in desoxycorticosterone and Compounds 2 to 6 exhibits the strongest reducing properties.

TABLE I

*Optical Density of Molybdenum Blue Produced by 0.303 mμ of Steroids after
Periods of Heating of 1 and 3 hours*

Arranged in order of density at 3 hours.

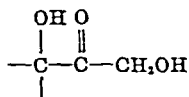
Compound No.	Common name	Structure	Log $\frac{I_0}{I}$	
			1 hr.	3 hrs.
1	Desoxycorticosterone	Δ^4 -Pregnen-21-ol-3,20-dione	0.69	0.90
2	“ ace-tate	“ ace-tate	0.45	0.88
3	Dehydrocorticosterone	Δ^4 -Pregnen-21-ol-3,11,20-trione	0.60	0.78
4	12-Ketodesoxycorticosterone acetate	Δ^4 -Pregnen-21-ol-3,12,20-trione acetate	0.38	0.74
5	21-Acetoxypregnenolone	Δ^5 -Pregnen-3(β),21-diol-20-one 21-acetate	0.33	0.69
6	21-Hydroxypregnenolone	Δ^5 -Pregnene-3(β),21-diol-20-one	0.57	0.68
7	Compound E (Kendall)	Δ^4 -Pregnene-17(β),21-diol-3,11,-20-trione	0.51	0.65
8	Methyltestosterone	17-Methyl- Δ^4 -pregnen-17(α)-ol-3-one	0.30	0.60
9	None	3(α),12-Dihydroxy-11-ketocholanic acid	0.33	0.54
10	“	Pregnane-3(α),12,21-triol-20-one 21-acetate	0.22	0.53
11	“	Cholestan-2(α)-ol-3-one acetate	0.19	0.51
12	Compound P (Reichstein) diacetate	Allopregnane-3(β),17(β),21-triol-20-one 3,21-diacetate	0.21	0.46
13	None	Cholestan-2(β)-ol-3-one acetate	0.15	0.42
14	Progesterone	Δ^4 -Pregnen-3,20-dione	0.21	0.38
15	None	Potassium 3(α),11(β)-dihydroxy-12-ketocholanoate	0.24	0.37
16	“	3(α),11(α)-Dihydroxy-12-ketocholanic acid	0.14	0.36
17	Ethinyltestosterone	17-Ethinyl- Δ^4 -androstene-17(α)-ol-3-one	0.17	0.31
18	cis-Testosterone	Δ^4 -Androsten-17(β)-ol-3-one	0.17	0.30
19	Androstenedione	Δ^4 -Androstene-3,17-dione	0.16	0.29
20	Cholestenone	Δ^4 -Cholesten-3-one	0.17	0.29
21	None	3(α)-Hydroxy-11,12-diketocholanic acid methyl ester	0.09	0.27
22	Testosterone	Δ^4 -Androsten-17(α)-ol-3-one	0.16	0.26
23	17-Hydroxypregnanolone	Pregnane-3(α),17-diol-20-one	0.03	0.12
24	7-Ketcholesterol acetate	Δ^4 -Cholesten-3(β)-ol-7-one acetate	0.03	0.09
25	Pregnenolone	Δ^5 -Pregnen-3(β)-ol-20-one	0.02	0.09
26	None	Δ^4 -Cholestene-3,6-dione	0.02	0.09
27	Pregnanedione	Pregnane-3,20-dione	0.02	0.08

TABLE I—*Concluded*

Compound No.	Common name	Structure	$\text{Log } \frac{I_2}{I}$	
			1 hr.	3 hrs.
28	α -Estradiol	$\Delta^{1,2,5}$ -Estratriene-3,17(α)-diol	0.00	<0.04
29	Estrone	$\Delta^{1,2,5}$ -Estratrien-3-ol-17-one		
30	Estriol	$\Delta^{1,2,5}$ -Estratriene-3,16,17,-triol		
31	Cholesterol	Δ^5 -Cholesten-3(β)-ol		
32	Allocholesterol-isoallocholesterol	Δ^4 -Cholesten-3(α and β)-ol		
33	None	3(α)-Acetoxy-12-keto- $\Delta^4,11$ -cholenic acid		
34	"	Cholestane-3(β), 7(α)-diol-6-one diacetate		
35	"	Cholestane-3(β), 7(β)-diol-6-one diacetate		

We are grateful to Dr. T. F. Gallagher for specimens of Compounds 9, 15, 16, and 21, to Dr. E. C. Kendall for Compounds 3, 7, and 33, and to Dr. S. Lieberman for Compound 23. Compound 32 was prepared by the method of Schoenheimer and Evans (4) and Compound 26 by the method of Mauthner and Suida (5). In this laboratory our thanks are due to Miss D. Sainte-Marie and Mr. H. Falk for the preparation of Compounds 4 and 10 by a modification of the method of Fuchs and Reichstein (6), and to Miss J. Cohen for the isolation of Compound 12 from an adrenal cortical fraction kindly provided by The Upjohn Company, through the courtesy of Dr. M. H. Kuizenga. The preparation of the cyclic α -ketols, Compounds 11, 13, 34, and 35, will shortly be described by one of us (R. D. H. H.) in collaboration with Dr. B. K. Wasson; in each epimeric pair the β configuration has tentatively been assigned to the isomer with the lower melting point and the more negative rotation. For supplies of Compounds 2 and 5, we are grateful to Dr. C. R. Scholtz, of Ciba Pharmaceutical Products, Inc., and to Dr. E. Schwenk, of the Schering Corporation.

Group B—The reducing power of the steroids of Group A is diminished slightly by the presence of a tertiary hydroxy group at C₁₇; *i.e.*, the



side chain of Kendall's Compound E (compare Compounds 7 and 3, and 12 and 6).

Group C—Acetylation of the primary alcoholic function of Groups A and B diminishes the rate of development of the blue color but not the intensity finally attained after a 3 hour period of heating (compare Figs. 3 and 1, and Compounds 2 and 1, and 5 and 6). Presumably hydrolysis of the acetate takes place slowly in the acid reaction medium to release the free ketol.

Group D—Substitution of a ketonic oxygen atom at C_{11} or C_{12} adversely influences the reducing capacity of the α -ketol side chain (compare Compounds 3 and 1, 4 and 2, and 10 and 5).

Group E—A tertiary α -ketol (Compound 23) is essentially non-reducing.

Group F—Cyclic secondary α -ketols in Ring A, as acetates (Compounds 11 and 13), and in Ring C, as free ketols (Compounds 9, 15, and 16), exhibit reducing power of the same order as that of the aliphatic primary α -ketols. Ring B ketols (Compounds 34 and 35), as acetates, fail to reduce, but the saponification product of Compound 35 (m.p. $202-203^\circ$) and the cholestane-3(β),7(?)-diol-6-one (m.p. 179°) described by Heilbron, Jones, and Spring (7) are strongly reducing; the inertness of the acetates, Compounds 34 and 35, is accordingly ascribed to failure of the acid medium to effect hydrolytic removal of the 7-acetoxyl groups.

In the case of both epimeric pairs studied (Compounds 11 and 13, and 15 and 16), there is an appreciable difference between the rates of reduction shown by the *cis* and *trans* isomers, which is still more marked in the case of the free ketols presumably corresponding to the acetates, Compounds 34 and 35. These preliminary observations strongly indicate that relative reducing power may prove valuable in the establishment of the configuration of secondary hydroxyl groups vicinal to a ketone in the steroid ring system; investigations in this connection are being extended.

Group G—An α,β -diketone (Compound 21) is moderately reducing.

Group H— α,β -Unsaturated 3-keto steroids, void of a ketol grouping in the molecule (Compounds 8, 14, 17, 18, 19, 20, and 22) show a reducing potential of the same order as the aliphatic and cyclic ketolic compounds not possessing the α,β -unsaturated 3-ketonic linkage. In the presence of both functions, the reducing power is approximately the sum of the two contributions.

Group I—Without reducing power are (a) α,β -unsaturated 7- and 12-keto steroids (Compounds 24 and 33), (b) Δ^4 - and Δ^5 -3-hydroxy compounds (Nos. 25, 31, and 32), and (c) the estrogens (Compounds 28, 29, and 30).

Exceptions to the above generalizations are to be found in the behavior of methyltestosterone (Compound 8) and Δ^4 -cholestene-3,6-dione (Compound 26). The former is much more strongly reducing than the other six α,β -unsaturated 3-ketones which were examined (Fig. 4), while the latter shows no appreciable reducing power. No explanation of the anomalous behavior of methyltestosterone is apparent. The non-reducing character of the doubly conjugated diketone (Compound 26) may be ascribed to the highly enolic nature of this system, which is evidenced by the ease with which enol esters are formed (5) and by the absorption spectrum data (Fig. 5). The main band in neutral ethanol ($\epsilon_{\max.} = 10,600$ at $251.5 \mu\mu$) is depressed and shifted toward the violet in the presence of alkali ($\epsilon_{\max.} \approx$

10,000 at 259 $m\mu$). Contrariwise, with a simple α,β -unsaturated 3-ketone such as cholestenone (Compound 20), which is moderately reducing, the position and intensity of the main resonance band ($\epsilon_{\max} = 15,000$ at 240 $m\mu$) are unaltered by the addition of alkali, but the secondary ketone band ($\epsilon_{\max} = 140$ at 290 to 296 $m\mu$) is shifted and depressed ($\epsilon_{\max} = 127$ at 302 $m\mu$). These facts are taken to indicate that Compound 20 exists in solution mainly in the keto and Compound 26 mainly in the enol state.

Recently Talbot *et al.* (8) have applied to the estimation of certain adrenal steroids Nelson's modification (9) of the classical Folin-Wu method

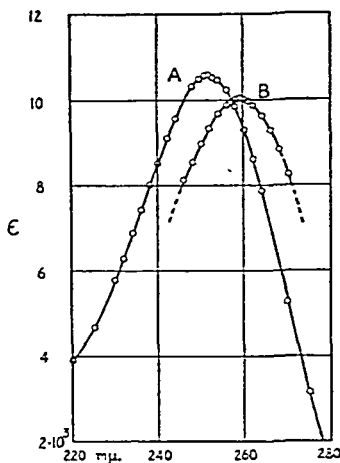


FIG. 5. Absorption spectrum of Δ^4 -cholestene-3,6-dione in neutral (Curve A) and alkaline (Curve B) ethanol. The scale of the ordinate represents the molecular extinction coefficient.

for the determination of blood glucose. In this procedure, cupric ion serves as the primary oxidizing agent and the resulting cuprous ion in turn reduces arsenomolybdic acid to the blue chromophore. In order to compare the specificity of the two methods, it was found necessary to alter the composition of the reagents used by Talbot *et al.* (8) because of the extreme insolubility of most steroids in the usual aqueous alkaline copper solution. Ethanol (50 per cent) served as solvent medium, and to prevent the precipitation of sodium sulfate from the Nelson copper reagent, this salt was omitted; reduction of cupric ion was indicated by the formation of the usual blue color with phosphomolybdic acid. Under these conditions, the aliphatic primary α -ketol and the Ring A cyclic α -ketol groupings are strongly reducing, while α,β -unsaturated 3-ketones are not. Negative, or

doubtfully positive, results were observed with compounds containing a cyclic α -ketol grouping in Ring C; insolubility in the medium may limit the reaction in some of these cases.

EXPERIMENTAL

Reagents—

Phosphomolybdic acid solution, according to Folin and Wu (3).

Glacial acetic acid, analytical reagent grade, distilled from chromic anhydride.

Phosphomolybdic acid reagent, a solution made up of equal volumes of phosphomolybdic acid solution and glacial acetic acid (the precipitate formed on addition dissolves on mixing).

Method of Color Development and Estimation—The following procedure was finally adopted after preliminary investigations led to satisfactory methods of control of certain variable factors (discussed below).

A solution (0.10 ml.) of the appropriate steroid in glacial acetic acid, of such concentration that the stated volume contains the reducing equivalent of approximately 100 γ of desoxycorticosterone, is introduced into the bottom of a micro test-tube (75 by 8 mm. internal diameter). The reagent (2.00 ml.) is then added, and, after mixture by shaking, the tube is placed in a boiling water bath (see below) for precisely 60 minutes. On removal, it is immediately cooled (30 seconds immersion in a beaker of cold water), and the contents are quantitatively transferred with 8.00 ml. of the reagent to one of a pair of optically matched Evelyn colorimeter tubes (internal diameter 2.0 cm.). On admixture, air bubbles form, which, because of the viscosity of the solution, require a period of 90 seconds to rise to the surface. The optical density is then read within the next 4 minutes at 650 to 660 $m\mu$ (Filter 660 with the Evelyn colorimeter; Filter 650 with the Lumetron colorimeter).

A blank determination with 0.10 ml. of glacial acetic acid and 2.00 ml. of reagent is run simultaneously and in the same way, which serves as the control for the adjustment of the colorimeter to zero optical density. In this a visible yellow coloration develops on heating, but it is not apparent after dilution to 10.1 ml. with the reagent. Compared to water, the blank solution absorbs very slightly at 650 to 660 $m\mu$, but the optical density remains constant regardless of the period of heating. Accordingly it is seemingly needless to carry out a control with every determination, but this is advisable as a safeguard against the contamination of the reagent with any substance which may reduce phosphomolybdic acid at the elevated temperature.

The limit of error in the development and reading of the color is approximately ± 2 per cent. Ten repeat estimations of aliquots (101 γ) of a solu-

tion of desoxycorticosterone gave optical densities of 0.67 (twice), 0.68 (six times), and 0.69 (twice).

Temperature Control during Development of Color—The extreme importance of maintaining a uniform thermal environment during the development of the color is well illustrated (Table II) by the significant differences in the intensity of the blue color observed on heating 0.303 mm of methyltestosterone with 2.00 ml. of the reagent (a) in a moderately boiling water bath (standard conditions) and (b) in a gently boiling bath.

Satisfactory control of this variable was achieved by maintaining a constant heat input into a constant volume bath, and by immersing the tubes to the same depth on all occasions. A steam-heated bath was employed,

TABLE II
*Temperature Control during Development of Color**

Time of heating min.	Optical density	
	Moderately boiling bath (standard conditions)	Gently boiling bath
15	0.08	0.07
30	0.17	0.14
45	0.24	0.20
60	0.30	0.27
90	0.39	0.36
120	0.48	0.46
180	0.60	0.56

* 0.303 mm of methyltestosterone; apart from the rate of boiling of the bath, all other conditions were maintained constant.

with the inlet needle valve always opened to the same aperture (maintenance of a moderate degree of boiling). The water input regulator was adjusted to keep the level 1 inch below the top enclosure (concentric rings) of the vessel, and the test-tubes were suspended from the latter by means of spring clothes-pins clamped close by the lip of the tube, which insured immersion in the boiling water of the lower portion of the tube to a depth of 1½ inches. Under these circumstances, numerous control estimations of desoxycorticosterone, made over a period of 2 years, consistently agreed with the original standard curve within ± 3 per cent. Closer agreement, if desired, may probably be realized by the use of a thermostatically controlled, electrically heated oil bath maintained at 100°.

Because of the wide variation of color intensity with time, and particularly with temperature, it follows that the standard curves and rate of reduction curves obtained under slightly different conditions in other laboratories may depart appreciably from those recorded here.

Influence of Nature of Phosphomolybdic Acid Reagent and of Various Diluents on Development and Stability of Color—While the Folin-Wu phosphomolybdic acid solution alone is suitable for estimation of those crystalline steroids which are moderately soluble in water, its application to the neutral fraction of urine leads to a cloudy solution which must be cleared by extraction with ether before the intensity of the blue color may be determined. By the solvent action of the acetic acid contained in the phosphomolybdic acid reagent, this difficulty is obviated and a more intense blue color results.

Table III illustrates the effect of various diluents on the intensity and stability of the color produced on heating 117 γ of desoxycorticosterone with 2.0 ml. of the phosphomolybdic acid reagent for 1 hour. Dilution with the same reagent is requisite to maximum color intensity and stability.

Construction of Standard Curves and of Rate of Reduction Curves—The standard curves (Fig. 2) were arrived at by ascertaining the optical density of the molybdenum blue color produced by the heating for 1 hour of the recorded amounts (each in 0.10 ml. of glacial acetic acid) of the appropriate steroid.

Each of the rate of reduction curves, illustrated in Figs. 1, 3, and 4 and recorded in Table I, was constructed from a series of seven determinations reached by heating for the periods of time indicated 0.303 mm of the appropriate steroid (dissolved in 0.10 ml. of glacial acetic acid). This quantity represents the equivalent of 100 γ of desoxycorticosterone.

Reference Standard—Desoxycorticosterone has been selected as the standard of reference because it is the most strongly reducing and most readily available adrenal cortical steroid.

Crystalline desoxycorticosterone, prepared by saponification of the acetate with KHCO_3 at room temperature (the method of Reichstein and von Euw (10)), has a melting point of 141.5–142.5°. On storage for several months in a stoppered vial, the melting point falls (softens at 131°, flows at 138–141°), commensurate with which there is a diminution in reducing power of about 3 per cent. One recrystallization from ether yields a product identical with the freshly prepared material in these respects. In glacial acetic acid solution at room temperature, there is detectable loss of reducing capacity within 1 to 2 weeks; kept frozen and tightly stoppered in the refrigerator, the solution remains stable for many months.

Reduction of Cupric Ion—The following modification of the procedure of Talbot *et al.* (8) was adopted qualitatively to compare the behavior of the various reducing steroids in the two methods.

The material (0.1 mg. in 1.0 ml. of redistilled ethanol) was introduced in a Folin-Wu tube and 1.0 ml. of alkaline copper reagent was added. The latter was prepared as described by Nelson (9), except for the inclusion of

sodium sulfate. After mixing, the contents of the tube were heated in the boiling bath for 20 minutes, cooled, treated with 2.0 ml. of phosphomolybdic acid reagent, and immediately diluted with ethanol (50 per cent) to 25 ml.

TABLE III
Effect of Diluent on Intensity and Stability of Color

Diluent (8.0 ml.)	Time after dilution*	Optical density
	min.	
Phosphomolybdic acid reagent	0	0.81
	1	0.81
	2	0.81
	3	0.81
	4	0.81
	5	0.81
	6	0.81
	10	0.81
Phosphomolybdic acid solution	0	0.70
	1	0.68
	2	0.68
	3	0.68
	4	0.68
	5	0.70
	7	0.70
	10	0.74
Acetic acid (50%)	0	0.75
	1	0.75
	3	0.75
	5	0.74
	7	0.73
	10	0.72
Water	0	0.57
	1	0.56
	3	0.55
	5	0.55
	7	0.54
	10	0.52

* Time zero is read after 90 seconds have elapsed for the clearance of air bubbles formed on admixture after dilution.

The blue color developed was then compared in the usual way against the reagent blank, similarly treated.

Compounds 1, 2, 5, 6, and 13 rapidly and strongly reduced cupric ion. Progesterone failed to react, and with Compounds 9, 15, and 16 essentially negative results were observed, but in these instances limited solubility in the medium renders the interpretation doubtful.

In support of the investigation we are grateful to the Committee on Research in Endocrinology of the National Research Council (Washington), the Associate Committee on Medical Research of the National Research Council (Ottawa), and the Faculty of Medicine of McGill University (Cooper Fund). Our thanks are due to Miss L. Groth for skilful technical assistance.

SUMMARY

1. The reduction of phosphomolybdic acid to molybdenum blue has been applied to the quantitative estimation of small quantities (10 to 100 γ) of reducing steroids of the adrenal cortical hormone class.

2. The reaction is given by steroids containing a primary or secondary (but not tertiary) α -ketol function, an α,β -unsaturated 3-ketone group, or both.

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THE NEUTRAL LIPIDE-SOLUBLE REDUCING SUBSTANCES OF URINE AS AN INDEX OF ADRENAL CORTICAL FUNCTION*

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The colorimetric method for the estimation of the reducing steroids, reported in the preceding communication (2), was explored with the aim of providing a simple chemical procedure for the determination of urinary "cortin," which might be applied as an index of adrenal cortical function. All of the known physiologically active adrenal cortical steroids contain (a) an α,β -unsaturated 3-ketone and (b) a primary α -ketol side chain at C₁₇. As both groupings are strongly reducing (2), and in the absence of either no appreciable biological activity is retained, it is reasonable to suppose that the active substance or substances of urine possess the same essential groupings, and that the neutral water-insoluble, lipide-soluble reducing substances of urine should be comprised, at least in part, of the active excretory products. The present communication describes the application of the chemical method to the appropriately fractionated urine extracts, and reveals a marked parallelism in cases of experimental and clinical adrenal hypo- and hyperfunction between urinary reducing capacity (2) and the power to cause deposition of glycogen in the liver of adrenalectomized mice (3).

Trial extractions of aqueous solutions of desoxycorticosterone and of urine containing added desoxycorticosterone showed that three extractions with ether removed only about 80 per cent of the hormone, whereas the use of ethylene dichloride or of ether-chloroform (4:1) resulted in quantitative recovery (Table II). Adoption of the latter solvent mixture was resorted to because of the troublesome emulsions encountered with ethylene dichloride. Phenols and acids are then removed in the usual way by washing with 0.1 N sodium hydroxide solution, and the residual neutral extract, after evaporation to dryness at low temperature, is sufficiently purified for the application of the previously described color test (2).

The hydrogen ion concentration at which the urine is extracted markedly influences the quantity both of the active and of the total reducing substances which is recovered. On acidification to Congo red (pH 1 to 2 approximately) and immediate extraction, the biological titer is approximately twice that obtained on processing an aliquot of the same urine at

* Preliminary accounts have previously appeared (1).

pH 7 (3). Likewise reducing power increases with decreasing hydron concentration (Table III), the value at pH 2.0 (glass electrode) being 2 to 3 times that at pH 7. Over the range pH 2.0 to 0.5, which has not yet been examined with respect to the behavior of the active material, there is a progressive increase in the amount of reducing substances removed. In more strongly acid solution, however, (approximately 0.75 N sulfuric acid) and by the application of heat, there is marked destruction of reducing capacity (Table IV); desoxycorticosterone, similarly treated in aqueous solution, loses much of its reducing power. These behaviors, in the presence of acid of varying concentrations, render imperative the arbitrary choice and rigid control of hydron concentration. In the practical procedure, pH 1.0 (glass electrode) has been adopted. Whether acidification of the urine effects hydrolysis of water-soluble conjugates, or simply admits of more complete extraction, is an important but unsettled question. Apparently the latter holds for the larger part of the reducing substances. In one experiment (Urine 3, Table III), urine which assayed 8 γ per 100 ml. on extraction at pH 7, and 20 γ on extraction immediately after acidification to pH 2.0, gave only 9 γ when an aliquot was acidified to pH 2.0 and then neutralized to pH 8.0 and processed.

With the object of correlating the reducing power of the neutral lipid-soluble fraction with adrenal cortical function, the excretion of reducing substances by the male dog was examined following (a) unilateral adrenalectomy,¹ (b) bilateral adrenalectomy, (c) the intravenous administration of cortical extract, and (d) the subcutaneous administration of anterior lobe extract. To avoid contamination from feces, urine was collected through a rubber tube surrounding the penis. The output of reducing substances is accordingly expressed in relation to creatinine excretion rather than to time, and is calculated as the equivalent of desoxycorticosterone (in micrograms) excreted per mg. of creatinine. The first experiment, designed to study the effect of adrenalectomy on urinary reducing power, was conducted before the importance of control of hydron concentration at the time of extraction was realized; unacidified urine was therefore processed throughout. Values over the control period ranged from 0.61 to 0.73 γ . With the removal of the left adrenal, a gradual decline to 0.23 γ occurred over 10 days, followed, during the succeeding 48 days, by a steady rise to 1.25 γ , which may possibly signify compensatory hypertrophy of the remaining adrenal. The second gland was then extirpated. To protect the animal against the stress of the operation, cortical extract (Connaught Laboratories, 15 ml. in all) was administered on the day of the operation and those immediately preceding and following. Intravenous glucose-saline solution was given daily until death. 4 hours after the last injection

¹ We are much indebted to Professor H. Selye for the performance of the operations.

of cortical extract, the urinary reducing power was 1.66 γ , and 12 hours thereafter, 0.50 γ . A steady fall then set in to a minimum value of 0.28 γ , reached on the 6th postoperative day, after which time the animal remained prostrate and would no longer accept food. During the next 72 hours, the output increased to 0.76 γ . No further specimens of urine could be obtained, and death resulted 7 days later in spite of efforts to revive the animal with cortical extract. Demonstrated is a diminution, by at least 66 per cent, in the excretion of reducing substances following ablation of each gland, and a marked rise after the administration of cortical extract.

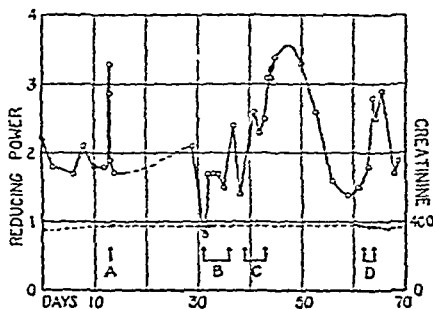


FIG. 1. The excretion of neutral lipid-soluble reducing substances by the intact dog, and following the administration of adrenal cortical and of anterior lobe extract. Scale of the left ordinate (solid curve) = urinary reducing substances, expressed as the equivalent of desoxycorticosterone (in micrograms) excreted per mg. of creatinine. Scale of the right ordinate (broken curve) = creatinine output in mg. per diem. Administration A = single intravenous injection of 70 ml. of cortical extract (reducing equivalent = 9.1 mg. of desoxycorticosterone). Administration B = subcutaneous injections over 6 days of 165 mg. of the product obtained from lyophilized anterior lobe on application of the method described by Reiss and Galla (6) for the extraction of corticotropin (see the text). Administration C = subcutaneous injections over 5 days of 2.4 gm. of lyophilized anterior lobe suspended in saline. Administration D = subcutaneous injections over 3 days of 1.4 gm. of lyophilized anterior lobe suspended in saline.

The apparent increased output 6 days after bilateral adrenalectomy may be attributed to reduced creatinine clearance and the development of acidosis, conditions which would be expected immediately before death, and which would lead to fallaciously high values when the output of reducing substances is ascertained from urine extracted at undetermined and variable hydron concentrations and computed in terms of creatinine excretion.

The relation between urinary reducing power and adrenal function is more strikingly illustrated in the intact animal following the intravenous supply of cortical hormones and following the stimulation of the adrenals with anterior lobe extract (Fig. 1). In this instance, all specimens of urine

were extracted at pH 1.0 and the daily creatinine output was shown to remain constant throughout. Excretion of reducing substances over the control periods ranged from 1.7 to 2.2 γ . Following a single intravenous injection of 70 ml. of cortical extract (Administration A, Fig. 1), containing the reducing equivalent of 9.1 mg. of desoxycorticosterone, the output at 3 and 6 hours was approximately twice normal (respectively 3.28 and 2.86 γ) but reached normal limits at 12 hours. The increased excretion over this period amounted to 0.31 mg., a recovery of 3.4 per cent of the equivalent administered. Higher recoveries, based on biological estimations, are reported in the literature; Harrop and Thorn (4) observed the excretion of 10 per cent of the activity (effect on salt and water metabolism) of an extract given intravenously to the dog, and Venning, Hoffman, and Browne (5) found that relatively large doses of extract administered intravenously to patients led to the elimination of 7 to 12 per cent of the active substances (cold exposure test) in the urine. After a rest period of 2 weeks, the effect of stimulation with hypophyseal extracts was examined. In the first instance (Administration B, Fig. 1), injected subcutaneously over 6 days was a total of 165 mg. of the product obtained from lyophilized anterior lobe² by the application of the method described by Reiss and Galla (6) for the preparation of purified corticotropin from fresh pituitary tissue. Irregular urine values followed. Subsequent bioassay of this preparation in the hypophysectomized rat³ by the method of Reiss *et al.* (7) showed a negligible corticotropin content. On the other hand, the administration (C, Fig. 1) of a saline suspension of 2.4 gm. of lyophilized whole anterior lobe² over a period of 5 days caused a slow and prolonged rise with a maximum output of 3.55 γ attained on the 9th day from the start of treatment. After return to the mean level, which required 17 days, the experiment was repeated at a lower dose level (1.4 gm.; Administration D, Fig. 1), with a similar response, less intense (maximum 2.9 γ) and less protracted (6 days).

With the experimental demonstration of correlation between urinary reducing power and cortical function, corroboration on clinical material was sought (Table I). Except in the determination of the normal values, aliquots of the same urine specimens were assayed by the chemical procedure and by the biological method of Venning, Kazmin, and Bell (3), the criterion of which is the deposition of glycogen in the liver of the adrenalectomized mouse. With the latter method, the results are expressed as the equivalent (in micrograms) of Kendall's Compound E excreted *per diem* (designated as "E" units).

Normal adult values were established from a series of single determinations, each on a different individual. The observed daily outputs of reduc-

² Kindly provided by Professor H. Selye.

³ Our thanks are extended to Dr. E. C. Hay for this assay.

ing substances in nine men were respectively 1.13, 1.19, 1.29, 1.34, 1.42, 1.44, 1.87, 2.05, and 2.06 mg. (average 1.53), and in nine women, 1.02, 1.04, 1.08, 1.15, 1.22, 1.29, 1.32, 1.92, and 2.01 mg. (average 1.34). By means of

TABLE I

Excretion of Reducing Substances and of Active "Cortin" under Physiological and Pathological Conditions

Subject	Reducing power		"Cortin"†	
		Ratio to normal†	Compound E per day	Ratio to normal†
	mg. per day*		γ	
Normal adult men	1.1-2.1		45-77	
Average (9 subjects)	1.53	1.0	62	1.0
Normal adult women	1.0-2.0		25-55	
Average (9 subjects)	1.34	1.0	39	1.0
Normal males, 2.5 yrs.	0.32	0.21	34	0.55
" " 3 "	0.47	0.31	42	0.68
" " 5.5 "	0.70	0.46	53	0.85
" " 7 "	0.79	0.52	58	0.94
Panhypopituitarism, Case 1 ♀	0.47			
" " 2 ♂	0.44	0.29	0‡	0
" " 2 ♂	0.62	0.40	0‡	0
Cushing's syndrome (♀)	4.80	3.6	212	5.4
Pregnancy, Case 1	2.48	1.9	111	2.8
" " 2	2.63	2.0	125	3.2
Burns,¶ Case I ♂, 4 days	3.0	2.0	121	2.0
" " " ♂, 24 "	2.4	1.6	63	1.0
" " " ♂, 45 "	0.84	0.55	55	0.89
" " " L ♂, 4 "	3.4	2.2	180	2.9
" " " ♂, 24 "	2.6	1.7	68	1.1
" " " ♂, 45 "	0.85	0.56	44	0.71
" " " C ♂, 4 "	2.7	1.8	102	1.6
" " " ♂, 24 "	1.2	0.78	44	0.71

* Expressed as the reducing equivalent of desoxycorticosterone.

† Calculated as the proportion of the average daily output of normal adult subjects of the appropriate sex.

‡ Compared by the power to cause deposition of glycogen in the liver of the adrenalectomized mouse (method of Venning, Kazmin, and Bell (3)), and expressed as the biological equivalent of Kendall's Compound E.

§ 8 hour excretion.

|| 9th month of gestation.

¶ First and second degrees; 25 to 40 per cent surface area. Time is recorded as the number of days following damage.

bioassay on different subjects it was found that ten males excreted respectively 65, 55, 55, 66, 45, 77, 70, 55, 70, and 60 "E" units *per diem* (average 62) and twelve females, 25, 35, 42, 33, 55, 25, 45, 40, 35, 35, 55, and 45

(average 39). The average output of women is thus lower than that of men (63 per cent, by bioassay; 88 per cent, by the chemical test), but the differences are not statistically significant. Also, by either criterion, the magnitude of the fluctuation in the daily excretion of the same individual on different occasions is of the same order as between different individuals of the same sex. One normal male subject gave reducing values of 1.80, 1.95, and 1.40 mg. *per diem* from December 14 to 17, 1944, and 1.18, 1.54, 1.30, 1.31, and 1.63 from January 2 to 6, 1945; variation similar in degree was encountered in women, with no apparent relation to the stage of the menstrual cycle. Based on glycogen deposition, the daily output of another normal male, assayed every other day for 2 weeks, was 46, 60, 49, 53, 58, 71, and 69 "E" units.

In the two cases of adrenal hypofunction (panhypopituitarism), the observed urinary reducing power is about one-third normal, while no glycogen activity was detectable in 8 hour urine specimens. On the assumption that adrenal cortical function is reduced to a minimum in these instances, it follows that a certain portion of the neutral lipide-soluble reducing substances of the urine is not of adrenal origin. In the unlikely event that no cortical function is retained by these subjects, the output of extraneous reducing substances is of the order of 0.5 mg. *per diem*; otherwise, the residual value must be somewhat lower. Likewise, in the adrenalectomized dog described above, the lowest value encountered after extirpation of the glands was approximately one-third of the normal value; however, the possible fallacies in the interpretation of the results of this particular experiment have already been pointed out.

Conversely, with adrenal hyperfunction, as in Cushing's syndrome and under the stress imposed by severe thermal damage and by gestation, the increased output of urinary "cortin" is accompanied by a commensurate and comparable rise in the excretion of reducing substances. By either test the observed increment is roughly 2 to 4 times the normal value (Table I).

Obviously it does not follow that a strict parallelism between the two criteria should prevail. Marked physiological activity, with respect to carbohydrate metabolism, is, in the light of present knowledge, dependent upon the presence in the steroid molecule of three functions, (a) the α,β -unsaturated 3-ketone grouping, (b) an alcoholic or ketonic oxygen atom substituted in the 11 position, and (c) the C_{20-21} α -ketol side chain, whereas reducing power is conferred by either (a) or (c) alone (2). Therefore it is conceivable that there may be many excretory products of cortical origin which retain power of reduction but which exhibit no biological activity. In instances of burns, Cases I and L, 24 days after damage, when the patients had not yet convalesced, the output of active material was normal,

while that of the reducing substances still remained high; after convalescence and rest (45 days), both determinations gave subnormal values. Conversely, with advancing age in children, the normal adult excretion of "cortin" is approached more rapidly than that of the reducing substances; this behavior and the changes at puberty are being investigated more fully.

Talbot *et al.* (8) have also examined the relation of urinary reducing power to cortical function. Their procedure differs from that described herewith in three respects: (a) the urine is not subjected to acid treatment prior to extraction with chloroform, (b) it is more completely fractionated, in that the total lipide-soluble neutral residue is partitioned between benzene and water⁴ before the final estimate is made on the ketonic components (separated with Girard's reagent) of the water-soluble portion, and (c) the reduction of cupric ion forms the basis of the colorimetric method. While in the data of Talbot *et al.* parallel biological estimations are not recorded, there is excellent general agreement between the results obtained by the two chemical methods. For normal subjects the Boston group finds an output of 0.12 to 0.34 (average 0.24) mg. *per diem* extractable from unacidified urine, as against averages of 1.3 (females) and 1.5 (males) extracted at pH 1.0, which are roughly 4 to 5 times the values obtained in processing at pH 7.0 (Table III). While the figures of Talbot *et al.* show no appreciable differences between sexes, it is our finding that the excretion of the female is lower than that of the male. In cases of adrenal hypofunction (Addison's disease and hypopituitarism), they record outputs of approximately one-half normal, which again suggests that a proportion of the neutral reducing substances of urine is of extra adrenal origin. Conversely, in cases of adrenal hyperfunction (Cushing's syndrome, virilism), and in the post-operative state, values several times normal are observed by either method.

EXPERIMENTAL

Reagents—

Ether—U. S. P. grade is distilled through a vertical column (90 cm. in height, and packed with 1 cm. glass collars), extracted twice with concentrated sulfuric acid (25 ml. to each liter of ether), and, without washing with water, again distilled. Without the sulfuric acid treatment, the quantity of ether employed in the extraction may give rise to an appreciable blank

⁴ The statement of Talbot *et al.* (8) that "only corticosteroids with an oxygen on the 11th carbon atom will tend to pass readily from benzene to water under the conditions used here" is not substantiated by their data (see also Mason, Myers, and Kendall (9), Reichstein and Shoppee (10), and Kuizenga (11)). While the $C_{21}O_4$ compounds, which include 17-hydroxycorticosterone and Compound E, are recovered quantitatively from the aqueous phase, so also is an 11-desoxy compound, Δ^4 -pregnene-17,20,21-triol-3-one. In the $C_{21}O_4$ series, corticosterone and dehydrocorticosterone remain mainly in the benzene (respectively 57 and 71 per cent).

color. Stored in the refrigerator, the ether remains stable for at least 2 weeks.

Chloroform—Analytical reagent grade is freshly distilled.

Ether-Chloroform—A 4:1 mixture of the two solvents is prepared immediately before the extractions are carried out. While a check for the presence of reducing substance in each batch of solvent is desirable, we have not encountered contamination in this respect when the ingredients have been purified as described above.

Precautions—Wash bottles used throughout the procedure are of all-glass construction.

No lubricant is employed with the glass stoppers and stop-cocks.

It is necessary that the rubber tubing in the nitrogen and vacuum lines be thoroughly scrubbed with hot alkali, washed with water, and dried before use. In each, a pledget of glass wool is inserted immediately adjacent to the rubber-glass connection to serve as a dust filter.

Extraction and Determination of Reducing Substances of Urine—An aliquot (100 ml. when a normal value is expected) of a 24 hour urine specimen (collected without preservative, stored in the refrigerator, and processed within the following 24 hours) is acidified by the cautious dropwise addition of 12 N sulfuric acid to pH 1.0 (Beckman glass electrode), and extracted once with 40 ml. of ether-chloroform and three times with 20 ml. portions of the same solvent mixture. To the combined extracts are added 40 ml. of ether-chloroform, which aids in the breaking of emulsions (centrifugation if necessary), and the solvent solution is then washed with chilled 0.1 N sodium hydroxide solution (five times with 10 ml. portions) and with water (5×10 ml. portions). Shaken with the organic phase in the separatory funnel is anhydrous sodium sulfate in quantity (approximately 1 to 1.5 gm.) just sufficient to absorb dissolved and adhering water; an excess of sodium sulfate should be avoided. The solution is then decanted and filtered through a pledget of clean (ether-washed) glass wool (contained in a conical transfer funnel) into a thick walled 300 ml. Erlenmeyer flask fitted with a standard taper ground glass joint. To effect quantitative transfer, the residual contents of the separatory funnel are shaken and rinsed twice with 10 ml. portions of ether, and the stopper, outer rim of the separatory funnel, and interior of the transfer funnel are well washed with a fine spray of ether from the wash bottle. The solvent is then distilled at low temperature in the vacuum of a water pump. For this purpose a standard taper male adapter fitted with a stop-cock in the vacuum line is employed; to avoid bumping the pressure is reduced gradually by the cautious opening of the stop-cock. When frost is apparent on the outside surface of the flask, the latter is immersed in a water bath at room temperature until evaporation is complete ($\frac{1}{2}$ to $1\frac{1}{2}$ hours). The residue is then transferred to a micro test-tube (75×8 mm. internal diameter) for the

development of the color. A fine spray of ether is played against the interior of the neck of the flask, while the latter is rotated three times. The solution (approximately 2.5 ml.) is then delivered to the test-tube with the aid of a thin glass rod, and the solvent is blown off at room temperature in a stream of nitrogen applied to the surface through a fine glass capillary tube. This transfer procedure is repeated three times, and finally the outer rim of the flask is washed with ether. To the total residue in the test-tube are added 2.00 ml. of the phosphomolybdic acid reagent, and the color is developed for a period of 60 minutes and read as previously described (2).

Recovery of Desoxycorticosterone from Water and from Urine—In Table II are given the recoveries of desoxycorticosterone added to water and to nor-

TABLE II
Recovery of Desoxycorticosterone from Water and Normal Male Urine

Desoxycorticosterone added*	Solvent	Volume	Reducing value†	Recovery
γ		ml.	γ	γ
100	Water	1000	100	100
100	"	1000	90	90
100	"	1000	104	104
100	"	1000	100	100
100	"	1000	94	94
0	Urine	500	99	0
100	"	500	202	103
0	" B	100	77	0
10	" "	100	86	9
0	" C	100	58	0
10	" "	100	71	13

* Contained in 0.1 ml. of acetic acid.

† Expressed as the desoxycorticosterone equivalent contained in the recorded volume.

mal male urine which were ascertained by the application of the extraction and colorimetric procedure described above. When the volume exceeds 100 ml., proportionally larger quantities of solvent, etc., were employed in the processing; the urines were extracted without previous acidification.

Effect of Hydron Concentration on Extraction of Reducing Substances from Urine—In Table III the results of the extraction of 100 ml. aliquots of different urines at various hydron concentrations (Beckman glass electrode) are given. Extractions were made immediately after acidification, except in the case of the third aliquot of Urine 3 which was allowed to stand at room temperature for 24 hours before being processed. The fourth portion of Urine 3 was acidified to pH 2.0, and then neutralized to pH 8.0 for extraction.

Action of Heat in Acid Medium, Strong Acid Treatment, and Periodic Acid on Reducing Substances of Urine—The experiments listed in Table IV were conducted on aliquot portions of the neutral fraction obtained on extraction

TABLE III

Effect of Hydrion Concentration on Extraction of Reducing Substances from Urine

Urine No.	pH	Reducing power
		γ per 100 ml.
1	7.0	9
	6.0	6
	4.0	12
	2.0	33
2	3.0	9
	2.0	22
	1.0	42
	0.5	80
3	7.0	8
	2.0	20
	2.0*	39
	8.0*	9

* For the conditions of extraction see the discussion in the text.

TABLE IV

Effect of Acid Treatment and of Periodic Acid on Reducing Power of Urine Extracts and of Desoxycorticosterone

Experi- ment No.	Material	Treatment	Assay
1	Urine extract	None	γ 164
		Boiled 10 min. in 15% HCl	51
2	" "	None	147
		Boiled 10 min. in 15% HCl	42
3	" "	None	96
		1 hr. in 2% H_2SO_4 (room temperature)	37
		1 " " 2% " and 3.5% HIO_4 (room temperature)	36
4	Desoxycortico- sterone (100 γ)	None	100
		1 hr. in 2% H_2SO_4 (room temperature)	76
		1 " " 2% " and 3.5% HIO_4 (room temperature)	4

at pH 1.0 of normal male urine. Different extracts were employed in the various experiments.

Boiling for 10 minutes in a medium containing 15 volumes per cent of

hydrochloric acid solution leads to destruction of at least two-thirds of the reducing substances present (Experiments 1 and 2). In these instances the urine residues were taken up in 50 ml. of hydrochloric acid (15 per cent), refluxed, cooled, and neutralized to pH 2.0 for extraction; the untreated control aliquots were simply suspended in 50 ml. of water, acidified to pH 2.0, extracted, and assayed.

Likewise marked loss of reducing power follows a period of 1 hour at room temperature in a medium containing 2 volumes per cent of sulfuric acid (Experiment 3). To a urine aliquot, assaying 96 γ , and contained in 0.3 ml. of methanol, was added 0.1 ml. of an 8 per cent solution of sulfuric acid in aqueous methanol (50 per cent). After 1 hour, the solution was diluted, extracted, and assayed. With periodic acid (14 mg.) in the same acid-methanol medium, there is no further destruction. On the other hand, desoxycorticosterone, treated similarly (Experiment 4), is practically quantitatively oxidized by periodic acid, while only 25 per cent of its reducing capacity is lost by the acid treatment alone.

Assay of Adrenal Cortical Extracts—The application of the above extraction and estimation to very dilute solutions (0.1 per cent) of cortical extract (Connaught Laboratories) gave extremely irregular results at all hydron concentrations. However, there does appear to be an increase in the amount of reducing substances extractable after acidification. By avoiding high dilution with water and the use of acid, reasonably consistent values could be obtained as follows.

To a dry separatory funnel, containing 75 ml. of anhydrous ether-chloroform (4:1), was pipetted 0.1 ml. of cortical extract, so that the solution was distributed over the interior surface of the funnel above the solvent. The mixture was then shaken into a single phase, washed three times with 5 ml. portions of cold 0.1 N sodium hydroxide, three times with water (5 ml. portions), and evaporated to dryness at room temperature for the development of the color. By this method 1 ml. of extract was found to contain 0.13 mg. of reducing substances.

In support of the investigation we are indebted to the Committee on Research in Endocrinology of the National Research Council (Washington), the Associate Committee on Medical Research of the National Research Council (Ottawa), and the Faculty of Medicine of McGill University (Cooper Fund). Our thanks are due to Mr. V. E. Kazmin and Miss L. Groth for skilful technical assistance.

SUMMARY

1. The reduction of phosphomolybdic acid to molybdenum blue has been applied to the estimation of the neutral, lipide-soluble reducing substances of urine.

2. Urinary reducing power and adrenal cortical function have been correlated. Following adrenalectomy in the dog, the excretion of reducing metabolites falls to approximately 33 per cent of the normal value, and following the administration of adrenal cortical or of anterior lobe extract to the intact animal, the output is increased 2 to 3 times.

3. In clinical cases of adrenal hypofunction (panhypopituitarism) and hyperfunction (Cushing's syndrome, and under stress), the daily excretion is respectively one-third normal and 2 to 5 times normal. Marked parallelism is observed in all instances between the output of reducing substances and that of biologically active metabolites which cause deposition of glycogen in the liver of the adrenalectomized mouse (urinary "cortin").

Addendum—It should be emphasized that the data embodied in the above text were obtained from urine specimens uncontaminated with blood or feces. Both blood and feces contain lipide-soluble reducing substances which seriously interfere with the determination as applied to the total neutral fraction of contaminated urine. Consequently, animal urine collected in the usual type of metabolism cage is quite unsuitable, and, for the same reason hospital specimens occasionally give fallaciously high values. Preliminary observations by Dr. Palmer Howard on the routine application of the method in a hospital laboratory indicate that further fractionation of the neutrals is requisite to a reliable estimate in instances of contamination.

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STUDIES ON THE MECHANISM OF ACTION OF THIOUREA AND RELATED COMPOUNDS

II. INHIBITION OF OXIDATIVE ENZYMES AND OXIDATIONS CATALYZED BY COPPER*

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The recent discovery of the rodenticidal properties of α -naphthylthiourea (ANTU) by Richter (1) has aroused interest in the mechanism by which this compound produces its acute toxic effects in animals. Factors associated with the toxicity of ANTU such as (a) the high toxicity to Norway rats, (b) the lower toxicity to most other species, (c) the development of tolerance after administration of sublethal doses, and (d) the severe pleural effusion and pulmonary edema produced by lethal doses are individually important to other toxic compounds. Information concerning the reactions undergone by ANTU may be of value in future studies on the mechanism of action and the factors which influence its toxicity to animals.

The biochemical changes which result in animals acutely poisoned by ANTU have been studied previously in this laboratory (2, 3) and it was found that marked hyperglycemia and depletion of liver glycogen occur in dogs and rats poisoned by this compound.

Several investigations on the effects of other thiourea derivatives on enzyme systems have been carried out in the hope of understanding their thyroid-inhibiting action. Enzymes included in these various studies were cytochrome oxidase (4, 5), succinic dehydrogenase (6), and tyrosinase (6, 7). The effect of monosubstituted derivatives of thiourea on oxidations catalyzed by copper (6) has also been studied. However, various investigators have obtained different results with the thiourea derivatives on these catalytic reactions. It was not possible, therefore, to predict the action of ANTU on these systems.

The present study was, therefore, undertaken to ascertain whether ANTU inhibits cytochrome oxidase and succinic dehydrogenase *in vitro* or *in vivo* after lethal doses of ANTU are given to rats. The effect of this rodenticide on tyrosinase and on oxidations catalyzed by inorganic copper was also measured. Since other thiourea derivatives which differ considerably in their toxicity to rats were available, it was of interest to com-

* This work was carried out under contract with the Medical Division of the Chemical Warfare Service.

pare their inhibitory action on these oxidative reactions *in vitro* with their toxicity to rats *in vivo*. The other compounds employed were allylthiourea, phenylthiourea, thiourea, and thiouracil.

EXPERIMENTAL

Methods and Materials

Tyrosinase activity was followed manometrically with a crude potato extract as the source of the enzyme. The extract was prepared by grinding potatoes in a Waring blender with one-third their weight of water and filtering. The filtrate retained most of its activity for 72 hours when stored at 10°. For the measurement of tyrosinase activity each Warburg vessel contained 0.2 ml. of the enzyme preparation, 0.3 ml. of 0.5 M phosphate buffer (pH 7.4), 4 mg. of *p*-cresol (in the side arm), and water to make a final volume of 3.0 ml. After a 10 minute equilibration period the substrate was tipped into the main compartment of the vessel. The reaction was carried out at 38°, and the oxygen consumption was recorded at 10 minute intervals for 30 minutes, during which time the rate of the reaction was linear with time and was limited by the tyrosinase concentration. When inhibitors were added, solutions of the compounds replaced water in the test system.

The oxidation of ascorbic acid was followed manometrically. Each Warburg vessel contained 5 mg. of ascorbic acid, 0.3 ml. of 0.5 M phosphate buffer (pH 7.4), 0.3 ml. of 1×10^{-5} M copper as cupric sulfate, and water to make a final volume of 3.0 ml. Oxygen consumption was recorded at 10 minute intervals for 30 minutes. The same test system was employed for measuring the oxidation of cysteine catalyzed by copper, except that 5 mg. of cysteine replaced the ascorbic acid and 0.3 ml. of 2×10^{-5} M copper as cupric sulfate was employed.

Succinic dehydrogenase and cytochrome oxidase activity were measured by the method of Schneider and Potter (8), with tissues from poisoned and normal adult Sprague-Dawley rats. Propylene glycol solutions of ANTU, phenylthiourea, and allylthiourea, and aqueous solutions of thiourea were injected intraperitoneally.

Results

Inhibition of Tyrosinase by ANTU and Related Compounds—To test the relative inhibitory action of ANTU and the other compounds on tyrosinase, various concentrations of the inhibitors were added to the tyrosinase test system. The inhibitors were incubated with the enzyme for 10 minutes before addition of the substrate from the side arm of the Warburg vessel. The effect of ANTU was compared with that of allylthiourea, thiourea, and phenylthiourea. Inhibition was calculated on the basis of the oxygen

consumption during the first 30 minutes of the reaction, as compared to a suitable control. The comparative effectiveness of ANTU, thiourea, phenylthiourea, and allylthiourea as inhibitors of the oxidation of *p*-cresol by tyrosinase is shown in Fig. 1, in which the tyrosinase activity is plotted against the negative logarithm of the inhibitor concentration. The control values are all expressed as 100, thus making it possible to include values obtained in different experiments with tyrosinase preparations of different activities.

The results of these experiments demonstrated that 50 per cent inhibition of tyrosinase was obtained with 8×10^{-4} M ANTU, 2.5×10^{-7} M phenylthiourea, 9×10^{-4} M allylthiourea, and 5×10^{-3} M thiourea. Thiouracil produced no inhibition at a concentration of 1×10^{-3} M. It is of interest to compare the effect of cyanide (9) with that of the thiourea derivatives

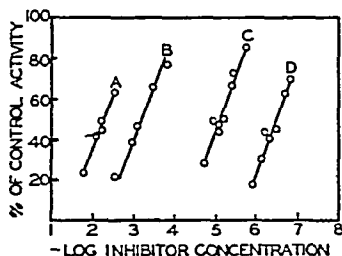


FIG. 1. The effect of thiourea and related compounds on tyrosinase activity. Curve A, thiourea; Curve B, allylthiourea; Curve C, α -naphthylthiourea; Curve D, phenylthiourea.

under the same experimental conditions. A final concentration of 1×10^{-4} M cyanide produced 50 per cent inhibition of tyrosinase activity.

Bernheim and Bernheim (6) have found that the inhibition of tyrosinase by phenylthiourea is reversible and varies with the substrate concentration. With concentrations of the compounds which would produce 50 per cent inhibition of tyrosinase, the reversibility of the inhibition and the effect of substrate concentration were studied. Under the conditions of our experiments there was no reversal of the inhibition by any of the compounds in 2 hours. Decreasing the amount of *p*-cresol in the test system from 4 to 2 mg. did not influence the inhibitory action of the compounds.

p-Cresol was employed as the substrate in all of these experiments because of the suitable rate of oxidation (10) of this compound for inhibitor studies. In order to ascertain whether the particular substrate employed influenced the inhibitory action of the thiourea compounds catechol, tyramine, and adrenalin were each substituted for *p*-cresol in the test

system. The test system was the same as that employed for *p*-cresol oxidation, except that tyramine (4 mg. per vessel), catechol (4 mg. per vessel), or adrenalin chloride (2 mg. per vessel) replaced *p*-cresol as the substrate. The oxidation of tyramine and adrenalin was measured for 30 minutes and the oxidation of catechol was measured for 5 minutes. The concentrations of inhibitors listed above which would produce 50 per cent inhibition of the oxidation of *p*-cresol by tyrosinase were employed. The oxidation of these three substrates was inhibited to the same extent as the oxidation of *p*-cresol by similar concentrations of ANTU, phenylthiourea, allylthiourea, and thiourea. The particular phenolic substrate employed, therefore, did not influence the inhibitory action of any of the compounds.

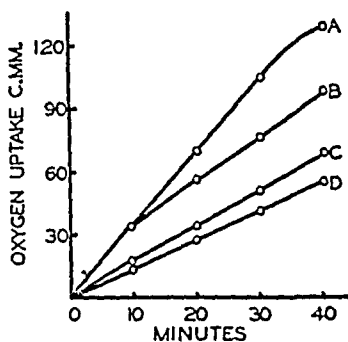


FIG. 2. The effect of varying the time of addition of α -naphthylthiourea (ANTU) and *p*-cresol to tyrosinase. Curve A, control; Curve B, ANTU added 10 minutes after *p*-cresol; Curve C, ANTU and *p*-cresol added at the same time; Curve D, ANTU added 10 minutes before *p*-cresol.

In all of the above experiments the inhibitors were incubated with the enzyme for 10 minutes before the addition of the substrate. To test whether the presence of the substrate protected the enzyme against inhibition by ANTU, the time of addition of the inhibitor was varied with relation to the time of addition of the substrate to the enzyme. The effect of adding ANTU to the enzyme system 10 minutes before the substrate, at the same time as the substrate, and 10 minutes after the addition of the substrate is shown in Fig. 2.

As may be seen from Fig. 2, the greatest inhibition (Curve D) was obtained when ANTU was incubated with the tyrosinase for 10 minutes before the addition of *p*-cresol. When the *p*-cresol and ANTU were added at the same time (Curve C), there was a small decrease in the amount of inhibition from 64 to 56 per cent. Addition of ANTU 10 minutes after the addition of *p*-cresol resulted in a further reduction to 44 per cent in the

amount of inhibition produced. The results indicate that the presence of the substrate affords some protection to the enzyme against inhibition by ANTU.

Certain substances which might reverse or prevent the inhibition of tyrosinase by ANTU were tested. Cysteine was tested because of the previous finding (2) that this sulfhydryl compound would protect rats against lethal doses of ANTU. In these experiments a final concentration of 1×10^{-4} M cysteine was ineffective in preventing or in reversing the inhibition of tyrosinase produced by 8×10^{-6} M ANTU. It has been reported that iodoacetic acid will overcome the inhibition of tyrosinase by thiourea derivatives (7). In our experiments, iodoacetic acid (1×10^{-3} M) was ineffective in preventing the inhibition when the iodoacetic acid and the ANTU were incubated for 10 minutes before addition to the enzyme, and it was also ineffective in reversing the inhibitory effects of ANTU on the enzyme.

Paschkis *et al.* (7) reported that the inhibition of tyrosinase by thiourea and thiouracil could be overcome by copper. It was of interest to test the effect of inorganic copper on the inhibition of tyrosinase by ANTU. For this experiment copper as cupric sulfate was employed with 8×10^{-6} M ANTU. A final concentration of 1×10^{-3} M copper was ineffective in reversing the inhibition of tyrosinase by ANTU or in preventing the inhibition when the ANTU and the copper were added to the enzyme at the same time. However, when the ANTU was incubated with the copper for 30 minutes at 38° before addition to the enzyme, then the amount of inhibition was reduced from 51 to 33 per cent.

The effect of iodine on the inhibition of tyrosinase by ANTU was also tested. Miller *et al.* (11) have recently demonstrated that thiourea derivatives react readily with iodine. If a similar reaction takes place between iodine and ANTU, then prevention of the inhibitory effects of ANTU on tyrosinase might be obtained with iodine.

This possibility was tested by incubating iodine and ANTU together in the side arm of the Warburg vessel for 10 minutes before addition to the tyrosinase in the main compartment of the vessel. The iodine was dissolved with the aid of twice its weight of potassium iodide. The potassium iodide itself in concentrations as high as 1×10^{-3} M had no antagonistic effect toward the inhibition of tyrosinase by ANTU. When a final concentration of 4×10^{-6} M iodine was incubated with ANTU (8×10^{-6} M) for 10 minutes before addition to the enzyme, the amount of inhibition was reduced from 46 to 30 per cent. Under these conditions an increase in the final concentration of iodine to 4×10^{-5} M afforded complete protection against inhibition of tyrosinase by 8×10^{-6} M ANTU.

The relative reactivity of iodine and tyrosinase with ANTU was studied

by adding the inhibitor and the iodine to the enzyme at the same time. A final concentration of 4×10^{-5} M iodine gave complete protection against inhibition of tyrosinase by 8×10^{-6} M ANTU when added simultaneously to the enzyme. This demonstrates a greater reactivity of ANTU with iodine than with tyrosinase. Iodine was ineffective in reversing the inhibition of tyrosinase by ANTU when the iodine was added to the test system 10 minutes after the addition of ANTU.

Effect of ANTU and Related Compounds on Oxidations Catalyzed by Copper—The antioxidant properties of thiourea and certain other related compounds have been well established. The ability of thiourea to inhibit the oxidation of ascorbic acid was reported by Fearon (12), and inhibition of the autoxidation of fats by thiourea and related compounds was recently shown by György *et al.* (13). From previous work it appeared that this inhibitory action may, in some cases, be due to a reaction between the thiourea compound and a metallic catalyst. In this connection it was of interest to test the effect of ANTU and related compounds on oxidations catalyzed by inorganic copper. In these experiments cysteine and ascorbic acid were employed as substrates.

Bernheim and Bernheim (6) found that the oxidation of cysteine catalyzed by inorganic copper was not inhibited by phenylthiourea and concluded that phenylthiourea has no effect on oxidations catalyzed by inorganic copper. We found that neither a final concentration of 1.4×10^{-2} M phenylthiourea nor a final concentration of 1.4×10^{-5} M ANTU had any effect on the oxidation of cysteine (5 mg. per vessel) as catalyzed by 2×10^{-6} M copper.

In contrast to the results obtained with cysteine, inhibition of the oxidation of ascorbic acid (5 mg. per vessel) as catalyzed by 1×10^{-6} M copper was obtained with ANTU, phenylthiourea, allylthiourea, and thiouracil. With this quantity of copper (1×10^{-6} M) the rate of oxygen consumption without added inhibitors was close to 35 c.mm. per 10 minutes. Inhibition was calculated on the basis of the oxygen consumption during the first 30 minutes of the reaction, as compared with a suitable control. The inhibitor was incubated with the copper for 10 minutes in the main compartment of the Warburg vessel before addition of the substrate from the side arm. The results of this experiment are shown in Fig. 3 in which the control values are expressed as 100 so that values obtained in different experiments can be included.

As shown in Fig. 3, all of the compounds inhibited the oxidation of ascorbic acid as catalyzed by 1×10^{-6} M copper. Allylthiourea was the least effective, a final concentration of 2.8×10^{-6} M being necessary in order to obtain 50 per cent inhibition of the reaction. A final concentration of 8.7×10^{-7} M ANTU, 1.0×10^{-6} M thiourea, 5.9×10^{-7} M phenyl-

thiourea, and 4.3×10^{-7} M thiouracil gave 50 per cent inhibition of the reaction.

The inhibitory action of these compounds was due to a reaction between the inhibitor and copper. This was demonstrated by varying the ascorbic acid and the copper concentrations of the test system containing a final concentration of 1×10^{-6} M ANTU. Variation in the ascorbic acid concentration had no influence on the amount of inhibition obtained by 1×10^{-6} M ANTU. An increase in the copper concentration to 1×10^{-5} M resulted in no inhibition of the reaction as compared with 50 per cent inhibition produced by 1×10^{-6} M ANTU when a lower concentration (1×10^{-6} M) of copper was employed.

Effect of ANTU and Related Compounds on Cytochrome Oxidase and Succinic Dehydrogenase—The recent demonstration (4) that the oxygen consumption of thyroid slices *in vitro* is not inhibited by high concentra-

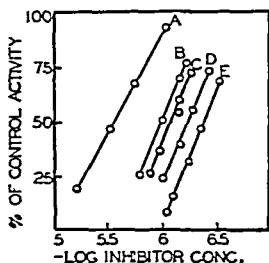


FIG. 3. The effect of thiourea and related compounds on the oxidation of ascorbic acid catalyzed by copper. Curve A, allylthiourea; Curve B, thiourea; Curve C, α -naphthylthiourea; Curve D, thiouracil; Curve E, phenylthiourea.

tions of thiourea would indicate that this compound does not inhibit cytochrome oxidase or succinic dehydrogenase. However, it has been reported that thiouracil is an effective inhibitor of cytochrome oxidase (5). The counteracting effect of cysteine in ANTU poisoning suggested the possibility that these compounds might react with sulfhydryl groups of enzymes. The present study was therefore undertaken to ascertain whether ANTU and related compounds have an inhibitory action on cytochrome oxidase and succinic dehydrogenase *in vivo* or *in vitro*.

Prior to enzyme studies, the toxicity of the compounds to rats was determined and the uniformly fatal doses were found to be 10 mg. per kilo of ANTU, 10 mg. per kilo of phenylthiourea, 400 mg. per kilo of allylthiourea, and 2000 mg. per kilo of thiourea. These quantities of the compounds produced death in 6 to 48 hours after injection with marked hyperglycemia occurring soon after injection of the drugs. Pulmonary

edema appeared about 5 hours after administration of the compounds. For the enzyme studies five rats were included in each group. The animals were sacrificed and lung and liver tissue was removed for the enzyme determinations at various times after administration of ANTU and the other compounds. There was no decrease from the average normal QO_2 of 88 in succinic dehydrogenase activity of liver tissue from poisoned rats in 3, 5, or 12 hours after ANTU. There was some decrease in the activity of lung tissue from a QO_2 of 12.4 for the normal animals to 9.0 for the poisoned group. Similarly there was no decrease in the succinic dehydrogenase activity of liver in 6 hours after allylthiourea, thiourea, and phenylthiourea. There was a slight decrease in the activity of lung tissue in 6 hours but in none of the groups did the QO_2 decrease to below 9.0. There was no change in the cytochrome oxidase activity of liver or lung tissue 6 hours after ANTU was administered to rats.

TABLE I

Effect of α -Naphthylthiourea and Related Compounds on Succinic Dehydrogenase and Cytochrome Oxidase Activity in Vitro

Inhibitor	M concentration	Succinic dehydrogenase, per cent inhibition		Cytochrome oxidase, per cent inhibition	
		Liver	Lung	Liver	Lung
α -Naphthylthiourea.....	0.000025	11	8	0	0
Phenylthiourea.....	0.0147	58	94	0	0
Allylthiourea.....	0.082	32	79	46	49
Thiourea.....	0.165	54	100	22	49

The effect of these thiourea derivatives on cytochrome oxidase and succinic dehydrogenase *in vitro* was also determined. The results of these measurements are summarized in Table I.

None of the compounds were effective inhibitors of either of these enzymes *in vitro*, as may be seen from the data in Table I. Saturation of the test system with ANTU or phenylthiourea produced no inhibition of cytochrome oxidase. Succinic dehydrogenase was inhibited to a greater extent than cytochrome oxidase by similar concentrations of the compounds. The succinic dehydrogenase of lung tissue was inhibited to a greater extent than that of liver by similar concentrations of the inhibitors.

DISCUSSION

These studies of the effects of ANTU on tyrosinase demonstrate that this new rodenticide is an effective inhibitor of that oxidative enzyme. Comparison of the relative inhibitory action of thiourea, ANTU, phenylthiourea, and allylthiourea on tyrosinase indicates that there is no cor-

relation between the effectiveness of the compounds as inhibitors of the synthesis of thyroxine (14) and their effectiveness as inhibitors of tyrosinase. Although no important rôle has been assigned to tyrosinase in mammalian tissues, it is interesting to note that the most effective tyrosinase inhibitors possessed the highest toxicity to rats, and the least toxic compounds, thiourea and thiouracil, are the least effective inhibitors of this enzyme. These results suggest that further studies on the oxidative enzymes may be of value in explaining the acute toxicity of ANTU.

The protective action of iodine against the inhibitory action of ANTU on tyrosinase indicates that ANTU reacts more readily with iodine than with the enzyme. Iodine, however, will not reverse the enzyme-inhibitor complex after it is formed, as was shown by the ineffectiveness of iodine added to the test system after ANTU. The reactivity of ANTU and iodine shown here is in agreement with the recent finding of Miller, Roblin, and Astwood (11) who demonstrated that thiourea and related compounds react readily with iodine. These observations give support to the hypothesis that the reaction between iodine and thiourea compounds may be an important factor in the thyroid-inhibitory action of these compounds.

The protection against the inhibition of tyrosinase by ANTU which was obtained by incubating the inhibitor with copper before addition to the enzyme indicates that copper reacts with ANTU to render ANTU ineffective as an enzyme inhibitor. The inability of copper to protect against the inhibition when it was added to the enzyme at the same time as the inhibitor demonstrates the greater reactivity of ANTU with tyrosinase than with copper.

The inhibitory action of ANTU and related compounds on the oxidation of ascorbic acid catalyzed by inorganic copper demonstrates the ability of all of these compounds to inhibit copper-catalyzed reactions. That this inhibition was due to a reaction with copper was shown by prevention of the inhibition when the copper concentration was increased. There was no correlation between the effectiveness of the various compounds as inhibitors of this process and their toxicity to rats. In regard to the anemia and leucopenia (15) produced by continued administration of these compounds, it seems worth while to investigate the possibility that interference with copper metabolism is involved, in view of the importance of copper in hematopoiesis (16).

The inability of ANTU and phenylthiourea to inhibit the copper-catalyzed oxidation of cysteine is in agreement with similar results obtained by Bernheim and Bernheim (6) with phenylthiourea. A reaction between cysteine and the sulfhydryl form of thiourea has been described by Toennies (17). A similar reaction between the sulfhydryl form of ANTU and cysteine in the test system to form a mixed disulfide may explain the

inability of ANTU to inhibit the oxidation of cysteine as catalyzed by copper. Such a reaction seems probable in view of the protective effect (2) of cysteine against ANTU poisoning in rats which was previously observed in this laboratory.

Measurements of cytochrome oxidase and succinic dehydrogenase activity of lung and liver from poisoned animals indicate that neither of these enzymes is inhibited by lethal doses of any of the thiourea compounds employed in these experiments. The apparent decrease in activity of these enzymes, based on dry weight of tissue, in lung tissue of poisoned animals is probably due to the accumulation of plasma in the damaged lung tissue. The *in vitro* experiments indicate that the compounds are more effective inhibitors of succinic dehydrogenase than of cytochrome oxidase. However, the high concentrations of all of the compounds which were necessary to produce inhibition of these enzymes make it improbable that either of these enzymes is inhibited in acute poisoning by thiourea and related compounds.

SUMMARY

1. Phenylthiourea, α -naphthylthiourea, allylthiourea, thiourea, and thiouracil inhibit tyrosinase. The most effective inhibitors are the most toxic to rats. The inhibition was not influenced by the particular substrate employed. The inhibition can be prevented by iodine or copper but cannot be reversed by these substances.

2. ANTU and related compounds are effective inhibitors of the oxidation of ascorbic acid as catalyzed by inorganic copper. This inhibition can be prevented by increasing the copper concentration in the test system.

3. Phenylthiourea and ANTU do not inhibit the oxidation of cysteine as catalyzed by inorganic copper.

4. None of the thiourea derivatives studied inhibit the cytochrome oxidase or succinic dehydrogenase of lung or liver tissue of rats given lethal doses of the compounds. High concentrations of the compounds are necessary to inhibit these enzymes *in vitro*.

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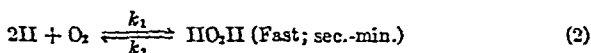
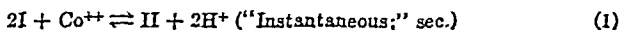
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16. Schultze, M. O., *J. Biol. Chem.*, **138**, 219 (1941).
17. Toennies, G., *J. Biol. Chem.*, **120**, 297 (1937).

LETTERS TO THE EDITORS

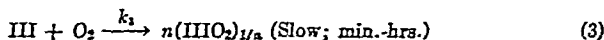
REVERSIBLE COMPLEXES OF COBALT, HISTIDINE, AND OXYGEN GAS

Sirs:

Cobalt has been found to be an effective inhibitor of growth and respiration of various aerobic and anaerobic microorganisms and animal tissues and tumors, and its physiologic action may be overcome reversibly and rather specifically by histidine among natural amino acids tested.¹ Titration and manometric (Warburg) experiments on the chemical mechanism of cobalt-histidine interaction indicate that, where I is $2(C_3H_7N_2)CH_2CH-(N^+H_3)CO_2^-$ and II is $[(C_3H_7N_2)CH_2(CHNH_2)CO_2^-]_2 Co^{++}$ ("colorless"),



(III, yellow-brown)



(IV, pink-red)

At 24° the equilibrium constant in (1) is $K_{1,0} = (II)(H^+)^2 / (Co^{++})(I)^2 = 4 \times 10^{-7}$. For example, at 0.01 M total histidine and equivalent total Co^{++} (0.005 M), (1) was 97 per cent complete at pH 7.5, 87 per cent at pH 6.5, and 74 per cent at pH 6.0. A maximum of 1 H^+ per charged amino group in I was also produced when either reactant in (1) was in excess rather than equivalent. The 2 protons coordinated on the amino groups of 2I in (1) are replaced by 1 Co^{++} in forming II, and are no longer titratable in the range of their normal pK, 9.2. With most amino acids, at 0.01 M, equivalent Co^{++} largely replaces the protons on the amino groups at pH values well above 7 or 8, near the amino pK values (thus, for example, with glutamic acid, glutamine, glycine, tryptophane, alanine, and proline, less than 10 per cent replacement at pH 6.0). The markedly lower pK of the imino group of histidine (6.0) permits double coordination of Co^{++} with histidine imino and amino N at lower, physiologically more important pH values, and provides a basis for the biological specificity reported earlier.¹

¹ Burk, D., Schade, A. L., Hesselbach, M. L., and Fischer, C. E., *Federation Proc.*, 5, 126 (1946). Burk, D., Hesselbach, M. L., Fischer, C. E., Hearon, J., and Schade, A. L., *Cancer Res.*, Abstracts, in press.

The equilibrium constant in (2), measured at pH 7.7, at which essentially all of the histidine occurs as II and III, was $K_2 = (\text{III})/(\text{O}_2 \cdot \text{aq})(\text{II})^2 = 3.1 \times 10^7$ and 6.9×10^4 liters² per moles² at 14° and 45° respectively (half saturating gas pressures of 0.0011 and 0.84 atmosphere, respectively, at initial (II) = 0.019 M); whence $\Delta H = -31,900$ calories per mole of O_2 (aq) taken up in (2). At 14° and pH 7.7 k_1 was 1.5×10^3 liters² per moles² per minute, and k_2 was 5×10^{-5} min.⁻¹. (2) may be reversed rapidly and completely by lowering pH or $p\text{O}_2$ or raising the temperature, much as in the case of oxyhemoglobin, for which compound III may be regarded as a simple, in some respects unique, model.

The irreversible uptake of 1 additional molecule of O_2 in (3) required some hours at 30–40° ($k_3 = 27$ liters per mole per minute at 38° and pH 7.0), and oxidation of Co^{++} to Co^{+++} was not evidenced by requisite production of 1 equivalent of hydroxyl ion.

Various other substituted histidine compounds, including carnosine² and anserine,³ behaved qualitatively like histidine, but Co^{++} was not observed to coordinate with 5-methylimidazole³ or N-acetyl glutamate.⁴

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² Kindly supplied by Professor Vincent du Vigneaud.

³ Kindly supplied by Professor D. Wright Wilson.

⁴ Kindly supplied by Dr. Otto K. Behrens.

A SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF DEHYDROPEPTIDASE ACTIVITY

Sirs:

Peptides of dehydroalanine (α -aminoacrylic acid) are split by dehydropeptidases in extracts of tissues to yield products including ammonia and pyruvic acid.¹ Dehydropeptidase activity has been estimated by noting the rate of formation of these products. An alternative method of following this activity is based upon the high characteristic ultraviolet

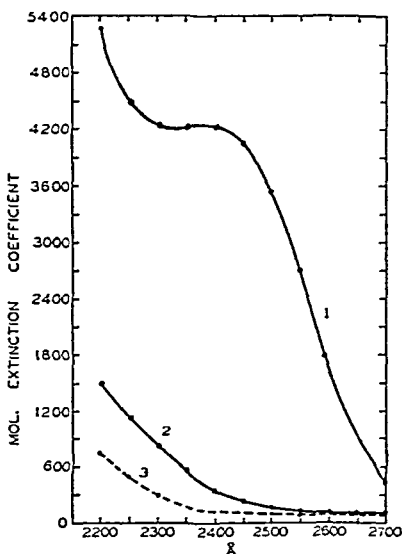


FIG. 1. Absorption curves in the ultraviolet of $1.7 \times 10^{-4} M$ aqueous solutions of (Curve 1) glycyldehydroalanine, (Curve 2) sodium pyruvate, and (Curve 3) glycylalanine.

absorption of dehydropeptides (Fig. 1). This absorption, absent in saturated peptide analogues, may be attributed to the tautomeric system

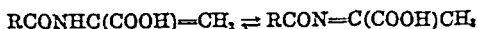


Fig. 1 illustrates the difference in ultraviolet absorption of equimolar solutions of glycyldehydroalanine and pyruvate. Other components of

¹ Bergmann, M., and Schleich, H., *Z. physiol. Chem.*, 205, 65 (1932); 207, 235 (1932). Greenstein, J. P., and Leuthardt, F. M., *J. Biol. Chem.*, 162, 175 (1946); *J. Nat. Cancer Inst.*, 6, 197 (1946).

the system have no appreciable absorption nor do they influence the characteristics of the dehydropeptide spectrum. Upon incubation of glycyldehydroalanine with tissue extracts, the absorption at 2500 A of the dehydropeptide decreases progressively, a finding attributable to the difference in absorption of glycyldehydroalanine and pyruvate at this wave-length. The final absorption of the extract plus products of the reaction is at the most 50 per cent of the absorption of the extract plus substrate at the beginning of the reaction.

The procedure employed follows. Tissues were ground with sand and extracted with 12 to 20 volumes of water; to 0.2 cc. of the centrifuged extract 0.2 cc. of a solution of glycyldehydroalanine (3.6 mg. per cc.) was added; following various periods of incubation at 37°, 10 cc. of distilled water were added to the digests and readings were made at 2500 A against a similarly treated extract blank (0.2 cc. of extract plus 0.2 cc. of H₂O). Under these conditions glycyldehydroalanine gave initial density readings of 1.4, which progressively decreased during incubation to approach a limiting value of nearly zero (complete hydrolysis). Activity may then be determined from the linear part of the hydrolysis curve in terms of mg. of substrate split per hour per mg. of total nitrogen in the extract used. Among the various tissues studied, kidney was found to have the highest activity, tumors of all kinds were uniformly high, and human sera exhibited a definite but low activity.

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THE INFLUENCE OF DIETARY PROTEIN, METHIONINE, AND CYSTINE ON ACCELERATED VITAMIN C EXCRETION IN THE RAT

Sirs:

King and coworkers¹ demonstrated that the vitamin C excretion of rats could be increased greatly by certain organic compounds and that this excretion probably represented an accelerated synthesis of the vitamin. The mechanism by which these agents act is obscure.

As the first step in the study of the mechanism of stimulated vitamin C excretion the effect of altering the dietary level of protein was determined. Adequately supplemented isocaloric diets containing 0.5 per cent choline in which only the protein and glucose levels were varied were fed to groups of four adult rats each (12 gm. per rat daily). After a 7 to 12 day preliminary period the stimulating agent, sodium phenobarbital or chloretone, was administered by stomach tube or in the diet. Determinations of 24 hour vitamin C excretion were made by the indophenol titrimetric procedure and checked colorimetrically.² The values were compared for the 7th through the 10th days after the first feeding of the stimulating agent, at which time the maximal excretion occurred. In all groups studied there was a significant increase in excretion of the vitamin over the consistently low daily average values of approximately 0.1 mg. per rat found during the preliminary period.

Increasing the level of casein from 0 to 5 per cent to 18 per cent resulted in a progressively increased ability of the rats to excrete vitamin C (1.74, 2.40, and 7.50 mg. per day per rat respectively) when fed 20 mg. of sodium phenobarbital daily. A further increase to 55 per cent resulted in a somewhat lower excretion (6.43 mg.). In another experiment increasing the level of arachin from 5 to 14 per cent also resulted in an accelerated excretion of the vitamin. Experiments were performed in which each of the ten essential amino acids and cystine were added to the 5 per cent casein diet; only cystine and methionine showed a marked accelerating effect on vitamin C excretion.

In the table are presented results of experiments in which the sulfur-containing amino acids were added to the 5 per cent casein diet to bring

¹ Musulin, R. R., Tully, R. H., 3rd, Longenecker, H. E., and King, C. G., *J. Biol. Chem.*, **129**, 437 (1939). Longenecker, H. E., Musulin, R. R., Tully, R. H., 3rd, and King, C. G., *J. Biol. Chem.*, **129**, 445 (1939). Longenecker, H. E., Fricke, H. H., and King, C. G., *J. Biol. Chem.*, **135**, 497 (1940).

² Robinson, W. B., and Stotz, E., *J. Biol. Chem.*, **160**, 217 (1945).

the total sulfur content up to that of an 18 per cent casein diet. Glycine was added in other experiments to supply nitrogen equal to that of the sulfur amino acids. Cystine and methionine increased the excretion of vitamin C to approximately the maximal level attainable with either of the stimulating agents under our experimental conditions, a level twice that obtained with

Influence of Supplementary Cystine and Methionine on Vitamin C Excretion of Rats on 5 Per Cent Casein Diet

20 mg. of sodium phenobarbital or 15 mg. of chloretone daily.

Amino acid added	Stimulating agent	Mean daily vitamin C excretion (days 7-10)
		<i>mg. per rat</i>
Glycine.....	Phenobarbital	3.52
Cystine + methionine*.....	"	6.98
Glycine.....	Chloretone	3.33
Cystine.....	"	6.17
Methionine.....	"	6.67

* In the proportions found in casein.

glycine. However, the addition of methionine to a milk diet supporting maximal vitamin C excretion depressed the excretion significantly.

These experiments demonstrate the important rôle of the sulfur-containing amino acids in regulating the capacity of the rat to synthesize and excrete large quantities of vitamin C in response to stimulating agents.

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THE EXCRETION OF NEUTRAL KETO COMPOUNDS AFTER INGESTION OF CARBOHYDRATE-RICH HEAT-PROCESSED FOODS*

Sirs:

Friedemann and Haugen¹ have shown that the urine of human subjects contains two groups of keto compounds: first, those which react rapidly, and, second, those which react very slowly, with 2,4-dinitrophenylhydrazine. It was later found that the first group consists principally of pyruvic and α -ketoglutaric acids. The daily excretion of each by seventeen normal men was characteristic and fairly constant for each subject regardless of the diet. The averages were as follows: pyruvic acid 20 ± 8 and α -ketoglutaric acid 33 ± 15 mg. per day. The excretion of the second group of keto compounds is variable, depending upon the content of indophenol-reducing substances ingested with heat-treated carbohydrate-rich foods. A high content of protein in such heated foods increases the excretion of the compounds. Physical activity has no apparent effect.

The compounds of the second group are not strongly acidic, since the 2,4-dinitrophenylhydrazones in organic solvents are not extracted by Na_2CO_3 solution. They have the following quantitatively parallel properties: (a) They react rapidly with sodium nitroprusside under the conditions of the Rothera test,^{2,3} yielding a violet color which reaches a maximum in 20 to 45 seconds, and then fades rapidly to brown or yellow within 3 to 4 minutes. This rapid reaction was first studied by Arnold⁴ and Holobut⁵ using the Legal reaction. The rapid fading is probably due to oxidation of the color complex by the nitroprusside (see (c) below). Sulfhydryl compounds react differently. (b) The substances react with 2,4-dinitrophenylhydrazine, the reaction requiring from 90 to 120 minutes under the conditions of the Lu⁶ and Friedemann and Haugen tests. (c) Like ascorbic acid, they are rapidly oxidized by 2,6-dichlorophenol indophenol, but the volume of indophenol reagent required for the titration

* This study was carried out under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Northwestern University. Additional funds were supplied by the Clara A. Abbott Fund of Northwestern University, Chicago, and the Nutrition Foundation, Inc., New York.

¹ Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, **147**, 415 (1943).

² Rothera, A. C. H., *J. Physiol.*, **37**, 491 (1903).

³ A quantitative method was devised for measuring the maximum color density.

⁴ Arnold, V., *Z. physiol. Chem.*, **49**, 397 (1906); **70**, 300 (1910-11); **83**, 304 (1913).

⁵ Holobut, T., *Z. physiol. Chem.*, **56**, 117 (1903).

⁶ Lu, G. D., *Biochem. J.*, **33**, 774 (1939).

is not materially changed in the presence of 8 per cent HCHO at pH 3 to 4.⁷ Properties (a), (b), and (c) indicate the following type structures:⁸ $\text{—COH=COH—CO—CH}_2\text{—}$ or —COH=COH—CO— . These structures, and the chemical properties noted, are similar in some respect to those of reductic acid and reductones, which are assumed to be present in carbohydrate-rich foods.⁹

The indophenol-reducing substances in foods do not give the nitroprusside reaction. However, they become reactive *after* ingestion of food. Thus, Arnold and Holobut noted that the urine of animals contains nitroprusside-reactive substances after oral or parenteral administration of extracts of heated foods. On the basis of present work, we believe that the parent substances in foods have the same structure as the metabolic derivatives, but that they are present in a bound form, perhaps combined with protein.

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⁷ Lugg, J. W. H., *Australian J. Exp. Biol. and Med. Sc.*, 20, 273 (1942). Snow G. A., and Zilva, S. S., *Biochem. J.*, 37, 630 (1943).

⁸ Friedemann, T. E., Sheft, B. B., and Miller, V. C., *Quart. Bull. Northwestern Univ. Med. School*, 20, 301 (1946).

⁹ Wokes, F., Organ, J. G., and Jacoby, F. C., *J. Soc. Chem. Ind.*, 62, 232 (1943).

THE ACTIVITY OF PYRIDOXAL PHOSPHATE IN TRYPTOPHANE FORMATION BY CELL-FREE ENZYME PREPARATIONS*

Sirs:

It has been known for some time that the tryptophane requirement of certain bacteria can be supplied by indole.¹ More recently Tatum and Bonner,² using a strain of *Neurospora*, have demonstrated the synthesis of

Tryptophane Formation by Cell-Free Enzyme Preparation

Enzyme from	Additions	Indole lost	Tryptophane formed	Indole lost	Tryptophane formed
		Micromoles $\times 10^2$, 1 hr.		Micromoles $\times 10^2$, 2 hrs.	
Fresh frozen mycelium	+ 0.44 μ M indole	3	2	5	4
	+ 0.44 " " + 1 mg. dl-serine	32	34	44	46
Mycelium held 7 days	Without additions		0		0
	+ 0.43 μ M indole	0	0	0	0
	+ 1 mg. dl-serine		0		0
	+ 0.43 μ M indole + 1 mg. dl-serine	6	13	18	21
	With 10 γ pyridoxal phosphate*		0		0
	+ 0.43 μ M indole	3	0	0	0
Mycelium held 32 days	+ 1 mg. dl-serine		0		0
	+ 0.43 μ M indole + 1 mg. dl-serine	14	19	31	34
	Without additions				
	+ 0.42 μ M indole	4	2	0	1
	+ 0.42 " " + 1 mg. dl-serine	5	5	6	6
	With 10 γ pyridoxal phosphate*				
	+ 0.42 μ M indole	0	0	0	0
	+ 0.42 " " + 1 mg. dl-serine	19	20	32	40

* 10 γ of the barium salt of pyridoxal phosphate, Sample 50-4.*

tryptophane from indole and serine. From this mold we have been able to prepare a cell-free system which converts indole plus serine into tryptophane. The enzyme has been resolved and pyridoxal phosphate found to

* Supported in part by a grant from the Nutrition Foundation, Inc.

¹ Fildes, P., *Brit. J. Exp. Path.*, 22, 293 (1941). Snell, E. E., *Arch. Biochem.*, 2, 389 (1943).

² Tatum, E. L., and Bonner, D., *Proc. Nat. Acad. Sc.*, 30, 30 (1944).

restore the activity. With the cell-free enzyme, indole disappearance³ was found to correlate with tryptophane formation.⁴

The cell-free enzyme may be prepared from the mycelium of *Neurospora sitophila* grown for 3 days, washed with distilled water, frozen, homogenized in 1.5 times its weight of 0.1 M phosphate buffer at pH 7.5, and centrifuged; 0.5 ml. of this preparation (\cong 5 mg. of protein), made up to 1 ml. with additions, was incubated at 37° (see the table).

If the frozen mold tissue is held several days before homogenization, the enzyme preparations obtained are much less active, but may be re-activated by the addition of pyridoxal phosphate.⁵ Since pyridoxal phosphate does not influence tryptophane formation in the absence of either serine or indole, its action appears to be in the indole-serine system leading to tryptophane.

These data provide evidence of another function of pyridoxal phosphate in addition to its rôle in amino acid decarboxylation⁶ and in transamination.⁷ The relationship of this function to the action of vitamin B₆ in tryptophane metabolism remains to be determined.⁸

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³ Stanley, A. R., and Spray, R. S., *J. Bact.*, **41**, 251 (1941).

⁴ Snell, F. D., and Snell, C. T., *Colorimetric method of analysis*, New York, **2**, 211 (1937).

⁵ Gunsalus, I. C., Umbreit, W. W., Bellamy, W. D., and Foust, C. E., *J. Biol. Chem.*, **161**, 743 (1945).

⁶ Gunsalus, I. C., Bellamy, W. D., and Umbreit, W. W., *J. Biol. Chem.*, **155**, 685 (1944). Braddily, J., and Gale, E. F., *Nature*, **155**, 727 (1945). Umbreit, W. W., and Gunsalus, I. C., *J. Biol. Chem.*, **159**, 333 (1945).

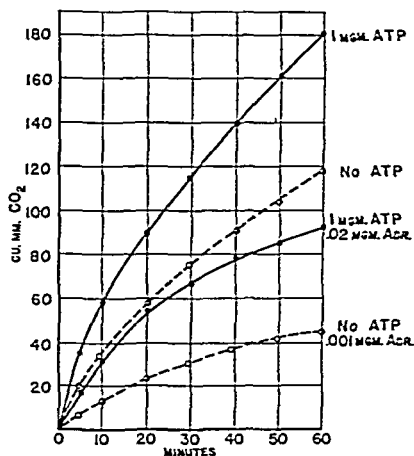
⁷ Lichstein, H. C., Gunsalus, I. C., and Umbreit, W. W., *J. Biol. Chem.*, **161**, 311 (1945). Green, D. E., Leloir, L. F., and Nocito, V., *J. Biol. Chem.*, **161**, 559 (1945).

⁸ *Nutr. Rev.*, **3**, 72 (1945).

THE INHIBITION OF THE ANAEROBIC GLYCOLYSIS OF RAT BRAIN BY ADRENOCHROME

Sirs:

The anaerobic glycolysis of rat brain homogenates has been found to be inhibited by small concentrations of adrenochrome. Adenosine triphosphate (ATP), which increases the rate of glycolysis, partially overcomes the inhibition due to adrenochrome. Glutathione, which has no effect on the glycolytic rate, abolishes the inhibitory effect of adrenochrome.



INHIBITION OF RAT BRAIN GLYCOLYSIS BY ADRENOCHROME

The manometric method of Utter, Wood, and Reiner¹ was used for measuring the glycolytic rate of rat brain homogenates. Additions of adrenochrome, ATP, or water were made to the substrate mixture to bring the volume to 1.5 ml. After allowing 5 minutes for equilibration of the reactants, the CO₂ production was measured over a period of 60 minutes. The materials, ATP, DPN, and HDP, were prepared by the methods described by Umbreit *et al.*² Adrenochrome was prepared by the following method:³ 1 gm. of epinephrine was dissolved in methyl alcohol con-

¹ Utter, M. F., Wood, H. G., and Reiner, J. M., *J. Biol. Chem.*, **161**, 197 (1945).

² Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric techniques and related methods for the study of tissue metabolism*, Minneapolis (1945).

³ U. S. Department of Commerce, Office of the Publication Board, Washington, D. C., Report No. 47 (1945).

taining 0.4 ml. of formic acid. 5 gm. of silver oxide (dry) were added and the mixture shaken for 1 minute. The mixture was filtered and the filtrate placed on dry ice for 1 hour. The crystals of adrenochrome were filtered off, washed with cold methyl alcohol, and dried under a vacuum.

In the absence of additional ATP, 0.001 mg. of adrenochrome inhibits the glycolytic rate by more than 50 per cent, whereas, in the presence of 1 mg. of ATP, 0.02 mg. of adrenochrome is necessary for 50 per cent inhibition. The inhibition of glycolysis by adrenochrome is not dependent on the concentration of DPN. The inhibition of glycolysis is reversed by glutathione; the inhibitory effect of 0.01 mg. of adrenochrome is abolished by 1 mg. of glutathione. Insulin is ineffective against the adrenochrome inhibition. The glycolytic rate is not inhibited more than 10 per cent by 0.002 to 0.2 mg. of epinephrine.

The part of the glycolytic cycle which is inhibited by adrenochrome has not been established. Since the degree of inhibition is not dependent on the concentration of DPN, it seems unlikely that systems involving this coenzyme are directly involved. Moreover, Green and Richter⁴ have shown that adrenochrome, formed during the oxidation of epinephrine by tissues, accelerates the rate of oxygen utilization by systems involving DPN as coenzyme (oxidation of lactic and malic acids). The fact that ATP partially overcomes the inhibition produced by small concentrations of adrenochrome suggests that the latter inhibits the phosphate transfer mechanism of the glycolytic cycle. Since oxidizing agents, such as iodine, quinone, and dichlorophenol indophenol produce an inhibition of glycolysis which is reversed by glutathione,⁵ it appears possible that adrenochrome also inhibits by a reversible oxidation of the sulfhydryl groups of the enzymes of glycolysis.

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Received for publication, August 8, 1946

⁴ Green, D. E., and Richter, D., *Biochem. J.*, **31**, 596 (1937).

⁵ Lipmann, F., in A symposium on respiratory enzymes, Madison, 65 (1942).

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MOLECULAR WEIGHT AND HOMOGENEITY OF CRYSTALLINE BOTULINUS A TOXIN

Sirs:

The isolation of *Clostridium botulinum* type A toxin has been reported.^{1,2} This crystalline toxin, the most potent known, contains about 220×10^5 mouse LD₅₀ per mg. of nitrogen.¹ In the equivalence zone both toxin and antitoxin are completely precipitated.

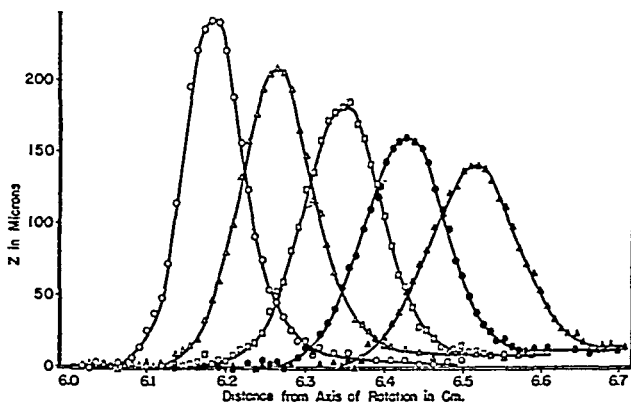


FIG. 1. Sedimentation velocity scale diagrams of crystalline botulinus A toxin; mean temperature, 32°; mean ultracentrifugal field, 48,000g; initial exposure at 30 minutes, interval between exposures 15 minutes; protein concentration, 0.17 per cent. Z denotes the scale line displacement.

The electrophoretic, sedimentation, and diffusion characteristics of the crystalline protein¹ have now been studied in 0.1 N sodium acetate buffer, pH 4.38. The toxin is electrophoretically homogeneous and has a mobility of 2.75×10^{-5} cm.² volt⁻¹ sec.⁻¹. The sedimentation diagrams of Fig. 1 show a single symmetrical boundary in the ultracentrifuge and yield a value of $S_{20} = 17.3$ Svedberg units. The diffusion constant of a 0.63 per cent solution measured at 25° by the refractometric scale method³ is 2.14×10^{-7} cm.² sec.⁻¹. Satisfactory agreement at successive time intervals among the values calculated by different methods and a good fit of the normalized diffusion curves with the ideal distribution curve have

¹ Lamanna, C., McElroy, O. E., and Eklund, H. W., *Science*, 103, 613 (1946).

² Abrams, A., Kegeles, G., and Hottel, G. A., *J. Biol. Chem.*, 164, 63 (1946).

³ Apparatus available by the courtesy of Dr. Hans Neurath.

been realized. The boundary spread in the ultracentrifuge is greater than that attributable to diffusion alone. Correlated studies of the particle size distribution in the ultracentrifuge and the electron microscope are planned.

Assuming a partial specific volume of 0.75, the molecular weight calculated from S_{20} and D_{20} is 900,000. This compares with the value 1,130,000 calculated from diffusion and viscosity data for toxin prepared by another method.⁴ From these preliminary data a tentative frictional ratio of 1.76 may be assigned. If the molecules are assumed to be prolate ellipsoids, this figure corresponds to an axial ratio, $b/a = 14.6$, hydration being neglected.

The high molecular weight of this toxin must be considered in any explanation of its pharmacological action. Nitrogen content and amino acid analysis indicate that it is a typical protein.⁵ A molecular weight of 900,000 suggests the presence of 2.1×10^7 molecules per LD_{50} .

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Received for publication, August 5, 1946

⁴ Kegeles, G., *J. Am. Chem. Soc.*, **68**, 1670 (1946).

⁵ Buehler, H. J., Bornor, D. H., Schantz, E. J., and Lamanna, C., *J. Bact.*, **51**, 571 (1946).

ANTITHIAMINE ACTIVITY OF PLANT MATERIALS

Sirs:

In the course of a series of investigations dealing with the influence of toxic plant materials on rats, a supply of bracken fern (*Pteris aquilina*) was obtained from an area in which a serious outbreak of "fern poisoning" had just occurred in cattle known to have consumed large amounts of fern. The material was air-dried at room temperature, ground, and incorporated into three different rat rations at a level of 40 per cent of the total ration.

Rats fed such rations gained weight for about 10 days and then lost weight rapidly until death occurred some 20 days later. Symptoms strongly suggestive of thiamine deficiency were observed in these rats. Although the several rations in which the fern had been incorporated were estimated variously to have contained from 0.2 to 0.6 mg. of thiamine per 100 gm., large doses of thiamine (0.5 mg. daily *per os*) were administered to rats showing severe anorexia, emaciation, and polyneuritis. Untreated rats showing these symptoms invariably died within several days. Rats given thiamine supplements, with one exception, recovered with such remarkable promptness that gains of 22 to 49 gm. for the 1st week were obtained. Gains for the 2nd week ranged from 23 to 37 gm. Rats which received supplementary thiamine from the beginning made good gains over a period of 5 weeks on rations containing 40 per cent powdered fern. To date four lots of fern, of different stages of maturity, collected from different localities during 1945 and 1946, have proved toxic to rats. The above observations are based on experiments involving 90 rats, twenty-nine of which variously received thiamine supplements and twenty-six of which were allowed to die as parallel controls.

It therefore appears that we have obtained substantial evidence for the existence of plant substances which possess marked antithiamine activity. The mode of this action has not been determined. We have not noted any decrease in the toxicity of air-dried fern when heated at 105° in air for 18 hours. The causative agent is essentially insoluble in ethyl ether and in acetone but appears to be slightly soluble in 92 per cent ethyl alcohol.

The application of these findings to "fern poisoning" in cattle remains obscure. The problem in cattle is complicated by possible intervention of the ruminal microflora which are responsible for a variety of synthetic

activities. The symptoms noted in rats are perhaps more suggestive of those reported in horses than those observed in ruminants.

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INHIBITORY ACTION OF CERTAIN AMINO ACIDS ON CHICKS RECEIVING NICOTINIC ACID-LOW DIETS*

Sirs:

Briggs¹ and Krehl *et al.*,² working with chicks and rats, respectively, have recently shown growth-inhibiting effects of tryptophane-low proteins (gelatin) when incorporated in synthetic diets low in nicotinic acid. In an attempt to determine the cause of this growth inhibition, we have fed pure

Group No.	Supplement to basal diet	Total No. of chicks	Average weight at 4 wks.	No. dead at 4 wks.	Per cent with black-tongue
			gm.		
1	5% gelatin	18	218	1	45
2	10% "	18	118	5	100
3	As Group 2 + 5 mg. % nicotinic acid	18	312	0	0
4	" " 1 + 2% glycine	6	188	1	67
5	" " 1 + 4% "	12	145	3	100
6	" " 4 + 5 mg. % nicotinic acid	6	314	0	0
7	" " 5 + 5 " % " "	6	300	0	0
8	" " 1 + 5% amino acid mixture*	12	131	5	100
9	" " 8 + 5 mg. % nicotinic acid	3	308	0	0
10	" " 1 + 2% glycine + 0.5% arginine	6	131	0	100
11	" " 10 + 0.5% alanine	6	118	1	100
12	" " 8 minus arginine and glycine	6	251	1	100

* Glycine 2.0, *dl*-aspartic acid 0.6, *dl*-alanine 0.5, *l*(+)-arginine hydrochloride 0.5, *l*(+)-glutamic acid 0.5, *l*(-)-leucine 0.2, *l*(+)-lysine hydrochloride 0.2, *dl*-phenylalanine 0.1, *dl*-serine 0.2, *dl*-valine 0.1, *l*(-)-tyrosine 0.1.

amino acids, alone and in combinations, simulating in most instances their relative occurrences in gelatin.

The experimental procedure and the basal diet used were the same as that reported by Briggs,¹ except that *dl*-methionine was substituted for *l*(-)-cystine.

That the feeding of glycine has growth-inhibiting properties under the conditions employed is evident (Groups 4 and 5). Furthermore, certain

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¹ Briggs, G. M., *J. Biol. Chem.*, 161, 749 (1945).

² Krehl, W. A., Sarma, P. S., and Elvehjem, C. A., *J. Biol. Chem.*, 162, 403 (1946).

other symptoms associated with nicotinic acid deficiency, namely chick blacktongue, diarrhea, perosis, and poor feed utilization, were also aggravated by the feeding of glycine. But most important is the complete prevention of these cachectic conditions by the feeding of nicotinic acid (Groups 6 and 7). As high as 6 per cent of glycine has been tolerated by chicks, provided sufficient nicotinic acid is contained in the diet.

The feeding of an amino acid mixture (Group 8) and combinations of some of the components of this mixture demonstrated that the growth-inhibiting effect of gelatin is not entirely due to glycine. Arginine and glycine together (Group 10), and especially arginine, glycine, and alanine in combination (Group 11), showed marked inhibitory action. The other amino acids of the mixture, even in the presence of alanine, apparently were not involved in the growth inhibition at the levels fed, although the incidence of blacktongue was increased. Again, as in the case of glycine, nicotinic acid completely overcame the ill effects of the amino acid mixture (Group 9). These results indicate that nicotinic acid is concerned, in some manner, with the metabolism of amino acids, especially glycine, arginine, and alanine.

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THE INFLUENCE OF α -KETO ACIDS ON THE DESAMIDATION OF AMINO ACID AMIDES

Sirs:

The relatively weak desamidation of glutamine and of asparagine in aqueous extracts of rat liver is considerably increased when pyruvate is

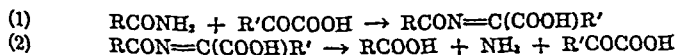
*Ammonia Nitrogen Evolved in Digests of Rat Liver with Various Substrates**

Substrate	Ammonia nitrogen
	7
Glutamine.....	9
" + pyruvate.....	102
" + pyruvoylglycinate.....	12
Glutamate.....	3
" + pyruvate.....	4
" + pyruvoylglycinate.....	4
Asparagine.....	22
" + pyruvate.....	135
" + pyruvoylglycinate.....	36
Aspartate.....	8
" + pyruvate.....	12
" + pyruvoylglycinate.....	9
Alanine.....	10
Alanylglycine.....	0
Pyruvate.....	1
Pyruvoylglycinate.....	0

* Mixtures consisted of 1 cc. of fresh aqueous tissue extract equivalent to 333 mg. of tissue plus 1 cc. of amino acid or amino acid amide plus 1 cc. of keto acid or 1 cc. of water. All substrates brought to pH 7.0 with NaOH before mixing. Initial pH 6.8. Concentration of amino acid and amide stock solutions 1.4×10^{-2} M, of the keto acids 2.7×10^{-2} M. Incubation period 4 hours at 37°.

The concentration of pyruvate alone and in the presence of the amides falls uniformly at the end of the incubation to 80 per cent of the initial value. The increase in free ammonia is accompanied by a corresponding decrease in amide nitrogen.

added to the mixture (see the table). We have provisionally interpreted the data in the table as being due essentially to two consecutive steps; namely, (1) a condensation of the amide with pyruvate to form a dehydropeptide, followed by (2) the splitting of the dehydropeptide by dehydropeptidase into products which include pyruvic acid and ammonia.



The net result of Reactions 1 and 2 is the desamidation of the amino acid amide and the recovery of the original keto acid. The latter thus

plays the rôle of a cosubstrate and is not appreciably altered in concentration throughout the digestion period (see the table).

Reaction 1 is that ordinarily employed in the *in vitro* synthesis of dehydropolypeptides.¹ Reaction 2 occurs in a wide variety of tissues.^{2, 3} Neither alanine nor alanylglycine yields much ammonia under the conditions used, which eliminates the possibility that transamination reactions may be concerned to any significant extent.

The failure of pyruvoylglycine to lead to such marked increase in ammonia production may be explained on the basis either of failure to condense with the amide, or of actual condensation with the amide to form a tripeptide in which the dehydroalanyl residue is in the middle of the chain and hence unreactive toward dehydropeptidase.²

Implicit in this concept is the formation of a peptide bond at the γ -carbon of glutamic acid and at the β -carbon of aspartic acid. The former type of bond is present in glutathione. Condensation of glutamine in the fashion indicated with pyruvoylglycine would lead to a desthiolglutathione, and work on this possibility is in progress.

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¹ Bergmann, M., and Grafe, K., *Z. physiol. Chem.*, **187**, 187 (1930).

² Bergmann, M., and Schleich, H., *Z. physiol. Chem.*, **205**, 65 (1932).

³ Greenstein, J. P., and Leuthardt, F. M., *J. Nat. Cancer Inst.*, **5**, 209 (1944); **6**, 197 (1946). Carter, C. E., and Greenstein, J. P., *J. Nat. Cancer Inst.*, **7**, in press (1946). Greenstein, J. P., and Leuthardt, F. M., *J. Biol. Chem.*, **162**, 175 (1946).

STUDIES ON THE EFFECTS OF PURINES ON METABOLISM*

Sirs:

It has been reported^{1, 2} that adenine or adenine plus monobasic sodium phosphate administered orally to dogs produces within a few weeks the syndrome of multiple avitaminosis (canine blacktongue) despite the normal vitamin-sufficient diet given to the dogs before and during the experimental period. These dogs also show an increase in blood urea nitrogen, non-protein nitrogen, liver and kidney damage, and a moderate rise in blood pressure. In similar experiments carried out on young albino rats it was found that the size and weight of the kidneys of the adenine-avitaminotic rats were much larger than kidneys of the controls.

Mice in groups of eight each, weighing between 19 and 21 gm., were kept on a normal diet supplemented with adenine or with adenine plus monobasic sodium phosphate for 1 to 3 weeks (adenine concentration 0.01 to 0.2 per cent; adenine plus phosphate concentration 0.01 to 0.2 per cent each). Sarcoma 180³ was then transplanted subcutaneously by the trocar method, care being taken to employ small fragments of uniform size. The animals were then kept on the same diet for 5, 8, or 14 days, when the tumors were compared with those of control mice. Other groups of mice (fourteen in each group) were placed on the same adenine or adenine plus phosphate diet on the day of tumor transplantation and kept on this diet during the tumor-bearing period (5, 8, or 14 days).

Under the conditions of these experiments a slight increase in the rate of growth of the tumor in mice fed adenine or adenine plus phosphate was observed when adenine either alone or with phosphate was administered at a concentration of 0.01 or 0.02 per cent in the normal diet for 1 to 3 weeks prior to or during the tumor-bearing period. Higher adenine concentrations (0.2 or 0.3 per cent) with or without added phosphate resulted in loss of appetite and retarded tumor growth.

Rats in groups of four, five, or six each, weighing between 120 and 170 gm., were placed on a normal diet supplemented by one of the following: either 0.06 per cent *p*-dimethylaminoazobenzene and 0.01, 0.02, 0.05, 0.1, or 0.2 per cent adenine or 0.06 per cent *p*-dimethylaminoazobenzene and 0.01, 0.02, 0.1, or 0.2 per cent adenine plus equal amounts of monobasic

* The work described in this paper was carried out under a research grant from the United States Public Health Service.

¹ Raska, S. B., *J. Am. Med. Assn.*, 131, 1093 (1946).

² Raska, S. B., *Science*, in press.

³ The tumor-bearing mice used for the tumor transplantations were kindly supplied by Dr. C. J. Kensler, Cancer Institute, Memorial Hospital, New York.

sodium phosphate. Other groups of rats (four or five in each group) were placed on a diet supplemented with adenine or adenine plus phosphate in corresponding amounts but without the dye. Six rats were kept on a diet containing 0.06 per cent of the dye without other supplement. The rats were kept on these diets for 38 to 69 days.

The pathological changes in the liver produced by the dietary intake of *p*-dimethylaminoazobenzene developed more rapidly and were more severe in the animals receiving the diet supplemented with 0.05, 0.1, or 0.2 per cent adenine or 0.05 to 0.1 per cent adenine and phosphate. However, it appeared that small amounts (0.01 to 0.02 per cent) of adenine in the diet either alone or with phosphate had a protective action against the liver damage produced by the dye.

It is probable that in the adenine-avitaminotic animal not only the rate of formation of a number of nucleoproteins and their split-products but also the function of various enzymes and coenzymes is altered.² Adenine in small concentration is essential for normal growth. Adenine or its metabolites in greater concentrations might be a significant factor in stimulating pathologic growth, possibly by inhibiting enzyme systems through interference with synthesis and utilization of coenzymes such as flavin-adenine dinucleotide, phosphopyridine dinucleotides (coenzyme I and II), and adenosine triphosphate.

Further studies are planned to determine the biochemical mechanism of the action of adenine. Studies with adenine containing isotopic carbon or nitrogen would appear to provide an ideal means for identification of the biological systems in which adenine plays a part, and for studies of the metabolism of adenine in health and disease.

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